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Den Haag, den
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Der Präsident des Europäischen Patentamts
Im Auftrag
For the President of the European Patent Office
Le Président de l'Office européen des brevets
p.o.

N. Mailliard

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V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NI NO NZ OM PH PL PT RO RU SC SD SE SG SK SL TJ TM TN TR TT TZ UA UG US UZ VC VN YU ZA ZM ZW
V-5	Precautionary Designation Statement In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.	
V-6	Exclusion(s) from precautionary designations	NONE
VI	Priority claim	NONE
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)

MULTIVALENT HEAVY CHAIN ANTIBODIES

FIELD OF THE INVENTION

The present invention provides multivalent single domain antibodies, comprising heavy chain antibodies, more precisely heavy chain antibodies composed of two or more single immunoglobulin variable domains, each domain having the same or different binding specificities directed to the same antigen, , the multivalent antibody having greatly improved affinity *in vitro* and *in vivo*. Homo-dimeric and homo-multimeric ligands and their uses are also described.

BACKGROUND OF THE INVENTION

Polypeptide therapeutics and in particular antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. Multivalency is one of the hallmarks of antibodies by which considerable gains in functional affinity can be achieved, both *in vivo* as well as *in vitro*. The principle of multivalency in the fight against pathogens is best illustrated by the natural immune response: the first molecule secreted in the immune response is IgM, containing between 10 and 12 binding sites (Randall *et al.*, JBC, 267, 18002-18007). Improving the intrinsic affinity of antibodies via somatic mutations retains the bivalency of antibodies.

Through the use of molecular biology techniques one can engineer multivalent antibody constructs with superior intrinsic and functional affinity. Such bivalent antibodies for instance may recognize different epitopes which can aggregate antigens, e.g. on a virus, more efficiently. This may lead to improved anti viral compounds.

Over recent years, single-chain Fv derivatives of antibodies have been developed in order to overcome limitation of the natural antibody format and enable the development of new formats such as fusion proteins. Also, such small antibody fragments can be produced in non-eukaryotic hosts such as *E. coli*. Compared to eukaryotic hosts, as required for the production of full antibodies, cloning and genetic manipulation is much more convenient in *E. coli*. However, the loss of multiple binding site per molecule usually results in a reduced affinity for the antigen as compared to the original, naturally bivalent, antibody. Multimer svFv's have been produced in order to overcome this problem (WO 9413806; WO9311161; US 5,989,830). These have been generated in many different ways: scFv's have been altered to include domains from IgG (Brocks et al, Immunotechnol 3, 173-184) or IgD heavy chain

constans region, helix-turn-helix proteins, p53 (Rheinnecker et al, J Immunol, 157, 2989-2997), IgG heavy- and light chains (Zuo *et al*, Prot Eng, 13, 361-367) or combination of these (Charles et al, Antimicrobial Agents Chemother 47, 1503-1508), a cystein residue in the C-terminus (Vallera et al, Blood 96, 1157-1165) or individual scFv domains have been concatenated with short linker peptides as spacers.

Roovers *et al* were able to obtain a 20-fold reduction in off-rate by dimerizing a scFv targeting Ep-CAM by fusing it to a human gamma1 hinge region and CH3 domain (Roovers *et al*, Cancer Immunol Immunther, 50, 51-59). Goel et al report a 4-fold increase in binding affinity constant when comparing a (sc(Fv)2)2 tetravalent reagent against the divalent sc(Fv)2 (Goel et al, Cancer Res, 60, 6964-6971). In contrast to the latter, Willuda and co-workers concluded tetramerization of a bivalent construct did not increase avidity of an anti-tumor scFv at all (Willuda et al., JBC, 276, 14385-14392). Thus, from this literature survey it appears that doubling the valency of scFv constructs can yield increases in binding constants from none at all to 20-fold. This increase in affinity, when present, translates into real therapeutic benefits as evidenced by the results of Goel (Goel *et al*, Clin Cancer Res, 7, 175-184; Goel *et al*, J Nucl Med 42, 1519-1527) or in diagnostic use, such as illustrated by Casey (Casey *et al*, Br J Cancer, 81, 972-980).

Antigen-binding proteins comprising heavy chain variable domains have been described in WO 0024884 and WO 9923221. In both cases the individual monomers are linked via a peptide linker, either constrained or not. However, the affinity of the antibodies so produced are no higher than would be expected from the addition of monovalent antibodies and no mention is made of the unexpectedly high functional affinities.

Bivalent single domain antibodies from *Camelidae* were also described in Conrath *et al* (JBC 276, 7346-7350). Individual domains were linked to each other via the long hinge of the llama IgG2a. Contrary to the finding of the invention, bivalent anti-lysozyme heavy chain VHH antibodies show a 4-5 fold avidity enhancement over the monovalent VHH molecule.

THE AIMS OF THE PRESENT INVENTION

It is an aim of the present invention to provide multivalent single domain antibodies, comprising heavy chain antibodies with improved functional affinity, which have superior performance compared to monovalent heavy chain variable domain antibodies when administered to a subject. It is a further aim of the present invention to provide methods for providing said multivalent heavy chain variable domain antibodies.

SUMMARY OF THE INVENTION

One embodiment of the present invention is a *Camelidae* multivalent VHH comprising two or more *Camelidae* VHHs directed against the same target.

Another embodiment of the present invention is a multivalent VHH as described above wherein said target is Tumour Necrosis Factor-alpha.

Another embodiment of the present invention is a multivalent VHH as described above wherein said two or more VHH correspond to sequences represented by any of SEQ ID NOs: 18 to 32.

Another embodiment of the present invention is a multivalent VHH as described above corresponding to the sequence represented by SEQ ID NO: 33 to 35.

Another embodiment of the present invention is a multivalent VHH as described above, wherein said multivalent VHH is a homologous sequence of said multivalent VHH, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a multivalent VHH as described above.

Another embodiment of the present invention is a multivalent VHH as described above, or a nucleic acid as described above for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

Another embodiment of the present invention is a use of a multivalent VHH as described above, or a nucleic acid as described above for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

Another embodiment of the present invention is a multivalent VHH of nucleic acid as described above or a use of a multivalent VHH as described above wherein said disorders are any of rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

Another embodiment of the present invention is a multivalent VHH or nucleic acid as described above or a use of a multivalent VHH as described above wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a multivalent VHH as described above wherein said target is vWF .

Another embodiment of the present invention is a multivalent VHH as described above wherein said target is collagen.

Another embodiment of the present invention is a multivalent VHH as described above wherein said two or more VHH correspond to sequences represented by any of SEQ ID NOs: 1 to 16.

Another embodiment of the present invention is a multivalent VHH as described above corresponding to the sequence represented by SEQ ID NO: 17.

Another embodiment of the present invention is a multivalent VHH as described above, wherein said multivalent VHH is a homologous sequence of said multivalent VHH, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a multivalent VHH as described above.

Another embodiment of the present invention is a multivalent VHH as described above, or a nucleic acid as described above for use in the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof.

Another embodiment of the present invention is a use of a multivalent VHH as described above, or a nucleic acid as described above for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof.

Another embodiment of the present invention is a multivalent VHH or nucleic acid as described above or a use of a multivalent VHH or nucleic acid as described above wherein said disorders are any of cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, and said conditions are those arising from coronary by-pass graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

Another embodiment of the present invention is a multivalent VHH or nucleic acid as described above or a use of a multivalent VHH as described above wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a multivalent VHH as described above wherein said target is Interferon-gamma.

Another embodiment of the present invention is a multivalent VHH as described above, wherein said multivalent VHH is a homologous sequence of said multivalent VHH, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a multivalent VHH as described above.

Another embodiment of the present invention is a multivalent VHH as described above, or a nucleic acid as described above for use in the treatment, prevention and/or alleviation of disorders wherein the immune system is over-active.

Another embodiment of the present invention is a use of a multivalent VHH as described above, or a nucleic acid as described above for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders wherein the immune system is over-active.

Another embodiment of the present invention is a multivalent VHH or nucleic acid as described above or a use of a multivalent VHH as described above wherein said disorders are any of Crohn's disease, autoimmune disorders and organ plant rejection in addition inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

Another embodiment of the present invention is a multivalent VHH or nucleic acid as described above or a use of a multivalent VHH as described above wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a composition comprising a multivalent VHH as described above, or a nucleic acid encoding said multivalent VHH and a pharmaceutically acceptable vehicle.

Another embodiment of the present invention is a composition comprising a multivalent VHH as described above, or a nucleic acid encoding said multivalent VHH and a pharmaceutically acceptable vehicle.

Another embodiment of the present invention is a composition comprising a multivalent VHH as described above, or a nucleic acid encoding said multivalent VHH and a pharmaceutically acceptable vehicle.

Another embodiment of the present invention is a composition comprising a multivalent VHH as described above, or a nucleic acid encoding said multivalent VHH and a pharmaceutically acceptable vehicle.

Another embodiment of the present invention is a method of diagnosing a disorder characterised by the dysfunction of Tumor Necrosis Factor-alpha comprising:

(a) contacting a sample with a multivalent VHH as described above,

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is a kit for screening for a disorder characterised by the dysfunction of Tumor Necrosis Factor-alpha, said kit comprising a multivalent VHH as described above and a standard.

Another embodiment of the present invention is a method as described above or kit as described above wherein said disorders are any of rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

Another embodiment of the present invention is a use of a polypeptide as described above for the purification of said Tumor Necrosis Factor-alpha.

Another embodiment of the present invention is a method of diagnosing a disorder characterised by the dysfunction of von Willebrand Factor comprising:

(a) contacting a sample with a multivalent VHH as described above,

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of von Willebrand Factor.

Another embodiment of the present invention is a kit for screening for a disorder characterised by the dysfunction of von Willebrand Factor, said kit comprising a multivalent VHH as described above and a standard.

Another embodiment of the present invention is a method as described above or kit as described above wherein said disorders are any of cerebral ischemic attack, unstable angina

pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, and those arising from coronary by-pass graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

Another embodiment of the present invention is a use of a polypeptide as described above for the purification of said von Willebrand Factor.

Another embodiment of the present invention is a method of diagnosing a disorder characterised by the dysfunction of Interferon-gamma comprising:

(a) contacting a sample with a multivalent VHH as described above,

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of Interferon-gamma.

Another embodiment of the present invention is a kit for screening for a disorder characterised by the dysfunction of Interferon-gamma, said kit comprising a multivalent VHH as described above and a standard.

Another embodiment of the present invention is a method as described above or kit as described above wherein said disorders are any of Crohn's disease, autoimmune disorders and organ plant rejection in addition inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

Another embodiment of the present invention is a use of a polypeptide as described above for the purification of said Interferon-gamma.

Another embodiment of the present invention is a multivalent VHH as described above wherein said target is involved in a disease process.

Another embodiment of the present invention is a multivalent VHH as described above, wherein said multivalent VHH is a homologous sequence of said multivalent VHH, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a multivalent VHH as described above.

Another embodiment of the present invention is a multivalent VHH a as described above, or a nucleic acid as described above for use in the treatment, prevention and/or alleviation of disorders or conditions in which the target is involved.

Another embodiment of the present invention is a use of a multivalent VHH as described above, or a nucleic acid as described above for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions in which the target is involved.

Another embodiment of the present invention is a multivalent VHH or nucleic acid as described above or a use of a multivalent VHH as described above wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a method of producing a polypeptide as described above comprising

(a) culturing host cells comprising nucleic acid capable of encoding a polypeptide as described above, under conditions allowing the expression of the polypeptide, and,
(b) recovering the produced polypeptide from the culture.

Another embodiment of the present invention is a method as described above, wherein said host cells are bacterial or yeast.

Another embodiment of the present invention is a multivalent single domain antibody comprising two or more a heavy chain immunoglobulin variable domains.

Another embodiment of the present invention is a multivalent antibody as described above comprising two or more variable domains directed against the same target.

Another embodiment of the present invention is a multivalent antibody as described above comprising two or more variable domains directed against the same or different epitopes on the same target.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the unexpected finding that multivalent single domain antibodies have functional affinities that are several orders of magnitude higher than the monovalent parent single domain antibodies. The inventors have found that the functional affinities of multivalent single domain antibodies are much higher than those reported in the prior art for bivalent and multivalent antibodies. Surprisingly, single domain antibodies linked to each other directly or via a short linker sequence show the high functional affinities expected theoretically with multivalent conventional four-chain antibodies.

According to the present invention, a specific class of single domain antibodies, known to the skilled addressee, are heavy chain variable domains derived from immunoglobulins naturally devoid of light chains such as those derived from *Camelids* as described in WO9404678 (and referred to hereinafter as VHH domains). VHHs are single polypeptides and very stable, resisting extreme pH and temperature conditions. Moreover, they are resistant to the action of proteases which is not the case for conventional antibodies.

The inventors have found that such large increased functional activities can be detected preferably with antigens composed of multidomain and multimeric proteins, either in straight binding assays or in functional assays, *e.g.* cytotoxicity assays.

A multivalent VHH as used herein refers to a polypeptide comprising two or more VHHs which have been covalently linked. The VHHs maybe identical in sequence or maybe different in sequence, but are directed against the same target or antigen. Depending on the number of VHHs linked, a multivalent VHH may be bivalent (2 VHHs), trivalent (3 VHHs), tetravalent (4 VHHs) or have a higher valency molecules.

The present invention relates to multivalent VHH comprising VHHs that belong to the all classes of *Camelidae* single domain heavy chain antibodies.

One embodiment of the present invention is a multivalent VHH comprising two or more VHH each directed against a therapeutic and/or diagnostic target. The multivalent VHHs disclosed herein have the advantage of unusually high functional affinity for the target, displaying much higher than expected inhibitory properties compared to their monovalent counterparts.

According to one aspect of the present invention, the VHHs of a multivalent VHH are linked to each other directly, without use of a linker. According to another aspect of the present invention, the VHHs of a multivalent VHH are linked to each other via a peptide linker sequence. Such linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. The linker sequence is expected to be non-immunogenic in the subject to which the multivalent VHH is administered. The linker sequence may provide sufficient flexibility to the multivalent VHH, at the same time being resistant to proteolytic degradation. A non-limiting example of a linker sequences is one that can be derived from the hinge region of VHHs described in WO 96/34103.

A subject according to the invention can be any mammal susceptible to treatment by therapeutic polypeptides.

According to an aspect of the invention VHHs used to form a multivalent VHH may be a complete VHH , a homologous sequence thereof, functional heavy or light chains derived from conventional antibodies and camelized versions thereof. According to another aspect of the invention, the VHHs used to form a multivalent VHH may be a functional portion of a complete VHH. According to another aspect of the invention, the VHHs used to form a multivalent VHH may be a homologous sequence of a complete VHH. According to another aspect of the invention, the VHHs used to form a multivalent VHH may be a functional portion of a homologous sequence of a complete VHH.

A homologous sequence of the present invention may include a polypeptide of the invention which has been humanised.

By humanised is meant mutated so that immunogenicity upon administration in human patients is minor or nonexistent. Humanising a polypeptide, according to the present invention, comprises a step of replacing one or more of the *Camelidae* amino acids by their

human counterpart as found in the human consensus sequence, without that polypeptide losing its typical character, *i.e.* the humanisation does not significantly affect the antigen binding capacity of the resulting polypeptide. Such methods are known by the skilled addressee.

Some VHH sequences displays a high sequence homology to human VH framework regions and therefore said VHH might be administered to patients directly without expectation of an immune response therefrom, and without the additional burden of humanisation. Therefore, one aspect of the present invention allows for the formation of a multivalent VHH without humanisation of the VHH, when said VHH exhibit high homology to human VH framework regions.

A homologous sequence of the present invention may include a sequence of the invention which in another *Camelidae* species such as, for example, camel, dromedary, alpaca, guanaco etc.

As used herein, a functional portion refers to a VHH or a multivalent VHH of sufficient size to sufficient size such that the interaction of interest is maintained with affinity of 1×10^{-6} M or better.

Alternatively a functional portion of a polypeptide of the invention comprises a partial deletion of the complete amino acid sequence and still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with the target or serum protein.

Delivery

One embodiment of the present invention is a multivalent VHH as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound intravenously or that is able pass through the gastric environment without being inactivated. As known by persons skilled in the art, once in possession of said a multivalent VHH, formulation technology may be applied to release a maximum amount of multivalent VHH in the right location (in the stomach, in the colon, etc.). This method of delivery is important for treating, prevent and/or alleviate the symptoms of disorder whose targets that are located in the gut system. An aspect of the invention is a method for treating,

preventing and/or alleviating the symptoms of a disorder requiring the delivery of a therapeutic compound that is able pass through the gastric environment without being inactivated, by orally administering to a subject a multivalent VHH specific for an antigen related to the disorder.

Another embodiment of the present invention is a multivalent VHH for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound to the vaginal and/or rectal tract. In a non-limiting example, a formulation according to the invention comprises multivalent VHHs directed against one or more targets in the form of a gel, cream, suppository, film, or in the form of a sponge or as a vaginal ring that slowly releases the active ingredient over time. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound to the vaginal and/or rectal tract, by vaginally and/or rectally administering to a subject a multivalent VHHs specific for an antigen related to the disorder.

Another embodiment of the present invention is a multivalent VHH for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound to the upper respiratory tract and lung. In a non-limiting example, a formulation according to the invention, comprises multivalent VHHs directed against one or more targets in the form of a nasal spray (e.g. an aerosol). Since multivalent VHHs are small, they can reach their target much more effectively than therapeutic IgG molecules. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound to the upper respiratory tract and lung, by administering to a subject said a multivalent VHH specific for an antigen related to the disorder by inhalation.

One embodiment of the present invention is a multivalent VHH for use in treating, preventing and/or alleviating the symptoms of disorders wherein the permeability of the intestinal mucosa is increased. Because of their small size, multivalent VHH can pass through the intestinal mucosa and reach the bloodstream more efficiently in subjects suffering from disorders which cause an increase in the permeability of the intestinal mucosa, for example Crohn's disease. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders wherein the permeability of the intestinal mucosa is

increased, by orally administering to a subject a VHH specific for an antigen related to the disorder.

One embodiment of the present invention is a multivalent VHH for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound that is able pass through the tissues beneath the tongue effectively. A formulation of said VHH, for example, a tablet, spray, drop is placed under the tongue and adsorbed through the mucus membranes into the capilliary network under the tongue. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound that is able pass through the tissues beneath the tongue effectively, by sublingually administering to a subject a multivalent VHH specific for an antigen related to the disorder.

One embodiment of the present invention is a multivalent VHH for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound that is able pass through the skin effectively. A formulation of said multivalent VHH, for example, a cream, film, spray, drop, patch, is placed on the skin and passes through. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound that is able pass through the skin effectively, by topically administering to a subject a multivalent VHH specific for an antigen related to the disorder.

TNF-alpha

According to one aspect of the invention, the target against which the VHHs of a multivalent VHH is directed is tumor necrosis factor alpha (TNF-alpha). TNF-alpha is believed to play an important role in various disorders, for example in inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

One aspect of the present invention relates to a multivalent VHH comprising two or more VHHs, the sequences of said VHHs corresponding to any of SEQ ID NOs: 18 to 32, derived from *Camelidae* heavy chain antibodies (VHHs), which bind to TNF-alpha.

The SEQ ID NOs in the present application refers to sequences in Table 1.

The aspect of the invention relating to anti-TNF-alpha VHH is not limited to polypeptides represented by SEQ ID NOs: 18 to 32, but may be extended to encompass polypeptides comprising *Camelidae* antibodies of any class directed towards TNF-alpha. Any of the multivalent VHHs disclosed herein may comprise a *Camelidae* antibody of any class . Said antibodies may be directed against whole TNF-alpha or a fragment thereof, or a fragment of a homologous sequence thereof. These antibodies include VHH, the full length *Camelidae* antibodies, and may comprise a human Fc domain if effector functions are needed.

One embodiment of the present invention is a multivalent VHH comprising two or more VHHs each directed against TNF-alpha for use in treating, preventing and/or alleviating the symptoms of inflammatory disorders. TNF-alpha is involved in inflammatory processes, and the blocking of TNF-alpha action can have an anti-inflammatory effect, which is highly desirable in certain disorder states such as, for example, Crohn's disease. Current therapy consists of intravenous administration of anti-TNF-alpha antibodies. Oral delivery of these multivalent VHH results in the delivery of such molecules in an active form in the colon at sites that are affected by the disorder. These sites are highly inflamed and contain TNF-alpha producing cells. These multivalent VHH can neutralise the TNF-alpha locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as *Micrococcus lactis* are able to secrete antibody fragments. Such modified microorganisms can be used as vehicles for local production and delivery of antibody fragments in the intestine. By using a strain which produces a TNF-alpha neutralising antibody fragment, inflammatory bowel disorder could be treated. Another aspect of the invention is a multivalent VHH comprising two or more VHHs each directed against TNF-alpha for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes, wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is the use of a multivalent VHH comprising two or more VHHs each directed against TNF-alpha for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to inflammatory processes, wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to inflammatory processes, comprising administering to a subject a multivalent VHH comprising

two or more VHHs each directed against TNF-alpha orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a multivalent VHH comprising two or more VHHs each directed against TNF-alpha for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes. Another aspect of the invention is a use of a multivalent VHH comprising two or more VHHs each directed against TNF-alpha for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

The above aspects and embodiments apply to a multivalent VHH comprising two or more anti-TNF-alpha VHHs wherein said VHHs correspond to any of SEQ ID NOs: 18 to 32, a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof. Examples of such sequences correspond to SEQ ID NOs: 33 to 35.

According to the Examples, antagonistic efficacy of VHHs and multivalent VHHs were analysed using a cell based assay, in which TNF-alpha induced cytostasis/cytotoxicity was determined by the calorimetric MTT assay as described by Vandenabeele and colleagues (Vandenabeele, P., Declercq, W., Vercammen, D., Van de Craen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W., Fiers, W. (1992).

The present inventors have shown in MTT assays (Figure 13) that the monovalent molecules used in this study had the poorest antagonistic characteristics, which is reflected by their IC50 values: the Fab derived from the chimeric antibody Remicade has an IC50 of 2 nM (not shown), and for multivalent VHHs of the present invention (*i.e.* comprising anti-TNF-alpha, VHH#2) it is 12 nM. The avidity of multivalent VHHs turns out to have a dramatic influence on the antagonistic efficacy as was observed with the bivalent IgG molecule Remicade, which is 40-fold more effective (IC50 50 pM) than the Fab. TNF-alpha is a trimeric molecule, which interacts to a dimeric receptor and therefore it can be expected that the avidity of the IgG permits the mutual binding to two epitopes on the cytokine and supports the formation of large complexes as has been described before (Santora et al, Anal.Biochem. 299, 119-129). Surprisingly, increasing the avidity of the multivalent VHHs compared with the monomer has a far more spectacular effect than observed with Remicade, since the IC50 of the dimer (30 pM) is 400 fold lower than of the monomer. Increasing the avidity even more leads to a still better antagonistic behaviour: the trimeric VHH has an IC50 of 20 pM and the tetravalent

format 6 pM. All higher avidity formats of the VHH are more efficient than Remicade, while the tetravalent VHH is even better than Enbrel, which consists of the extracellular domain of the receptor p75 fused to the Fc of an IgG and therefore has a bivalent binding mode.

The inventors have further demonstrated that the same unexpected effect of avidity on antagonistic behaviour was observed with VHH generated against mouse TNF-alpha (Figure 23). The same type of cytotoxicity assay was performed using MTT as substrate and mouse TNF-alpha (65 pg/ml or 1.3 pM), but with the murine cell line L929, which expresses the mouse specific receptor. Three different antagonistic (monovalent) VHH were identified code, 9E and 3F, of which the first two have IC50's of 25 nM and the latter 2 nM. Conversion of 3F into the bivalent format (BIV 3F, SEQ ID NO: 33) yielded a 1000 fold increase in IC50 (2 pM), thereby demonstrating once more that the increased avidity of the antibody leads to an unexpected improvement of the antagonistic characteristics.

vWF

Platelet-mediated aggregation is the process wherein von Willebrand Factor (vWF)-bound collagen adheres to platelets and/or platelet receptors, ultimately resulting in platelet activation. Platelet activation leads to fibrinogen binding, and finally to platelet aggregation. It is another aspect of the present invention to provide multivalent VHHs which modulate processes which comprise platelet-mediated aggregation such as, for example, vWF-collagen binding, vWF-platelet receptor adhesion, collagen-platelet receptor adhesion, platelet activation, fibrinogen binding and/or platelet aggregation. Said multivalent VHHs are derived from VHHs directed towards vWF, vWF A1 or A3 domains, or collagen.

According to one aspect of the invention, the target against which two of more VHHs of a multivalent VHH is directed is vWF. A1 or A3 domains. According to another aspect of the invention, the target against which two of more VHHs of a multivalent VHH is directed is gpIb. According to one aspect of the invention, the target against which two of more VHHs of a multivalent VHH is directed is gpIa/IIA. According to one aspect of the invention, the target against which two of more VHHs of a multivalent VHH is directed is collagen.

One aspect of the present invention relates to a multivalent VHH comprising two or more VHHs, the sequences of said VHHs corresponding to any of SEQ ID NOs: 1 to 16, derived from *Camelidae* heavy chain antibodies (VHHs), which bind to vWF.

The aspect of the invention relating to anti-vWF, anti-vWF A1 or anti-vWF A3, or anti-collagen VHHs. is not limited to polypeptides represented by SEQ ID NOs: 1 to 16, but may be extended to encompass polypeptides comprising *Camelidae* antibodies of any class directed towards vWF, vWF A1 or A3 domains or collagen. The VHHs disclosed herein may comprise a *Camelidae* antibody of any class, Said antibodies may be directed against whole vWF, vWF A1 or A3 domains or collagen or a fragment thereof, or a fragment of a homologous sequence thereof. These polypeptides include VHH, the full length *Camelidae* antibodies, and may comprise a human Fc domain if effector functions are needed.

One embodiment of the present invention is a multivalent VHH comprising two or more VHHs each directed against a target which is any of vWF, vWF A1 or A3 domains, or collagen for use in treating, preventing and/or alleviating the symptoms of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof. Said disorders include transient cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis. Said conditions include those arising from coronary by-pass graft, coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy. One aspect of the invention is a multivalent VHH comprising two or more VHHs each directed against a target which is any of vWF, vWF A1 or A3 domains, or collagen for use in the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof, wherein said VHH is administered intraveneously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is the use of a multivalent VHH comprising two or more VHHs each directed against a target which is any of vWF, vWF A1 or A3 domains or collagen for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders or conditions relating to relating to platelet-mediated aggregation or dysfunction thereof, comprising administering to a subject a VHH specific for an antigen related to the disorder or conditions orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a multivalent VHH comprising two or more VHHs each directed against a target which is any of vWF, vWF A1 or A3 domains or collagen

for use in the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof. Another aspect of the invention is a use of a multivalent VHH comprising two or more VHHs each directed against a target which is any of vWF, vWF A1 or A3 domains or collagen for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof. The anti-vWF, anti-vWF A1 or anti-vWF A3 or anti-collagen VHHs of the present invention may be derived from the new class of VHHs described above, or may be derived from any of the other classes of VHHs, including the major class of VHH.

The above aspect and embodiments apply to a multivalent VHH comprising two or more anti-vWF VHHs wherein said VHHs correspond to any of SEQ ID NOs: 1 to 16, a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

IFN-gamma

According to one aspect of the invention, the target against which two or more VHHs of a multivalent VHH is directed is interferon-gamma (IFN-gamma). IFN-gamma is secreted by some T cells. In addition to its anti-viral activity, IFN-gamma stimulates natural killer (NK) cells and T helper 1 (Th1) cells, and activates macrophages and stimulates the expression of MHC molecules on the surface of cells. Hence, IFN-gamma generally serves to enhance many aspects of immune function, and is a candidate for treatment of disorders where the immune system is over-active *e.g.* Crohn's disease, autoimmune disorders and organ plant rejection in addition inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

One aspect of the present invention relates to a multivalent VHH comprising two or more VHHs, the sequences of said VHHs corresponding to any resulting from our future experiments, derived from *Camelidae* heavy chain antibodies (VHHs), which bind to INF-gamma.

The aspect of the invention relating to anti-IFN-gamma VHH is not limited to polypeptides resulting from our future experiments, but may be extended to encompass polypeptides

comprising *Camelidae* antibodies of any class directed towards IFN-gamma. The any of the polypeptides disclosed herein may comprise a *Camelidae* antibody of any class, of the traditional class or of the new class of human-like *Camelidae* antibodies as disclosed herein. Said antibodies may be directed against whole IFN-gamma or a fragment thereof, or a fragment of a homologous sequence thereof. These polypeptides include the full length *Camelidae* antibodies, namely Fc and VHH domains.

One embodiment of the present invention is a multivalent VHH comprising two or more VHHs each directed against IFN-gamma for use in treating, preventing and/or alleviating the symptoms of the disorders wherein the immune system is overactive, as mentioned above. Current therapy consists of intravenous administration of anti-IFN-gamma antibodies. Oral delivery of these multivalent VHH results in the delivery of such molecules in an active form in the colon at sites that are affected by the disorder. These sites are highly inflamed and contain IFN-gamma producing cells. These multivalent VHH can neutralise the IFN-gamma locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as *Micrococcus lactis* are able to secrete antibody fragments. Such modified microorganisms can be used as vehicles for local production and delivery of antibody fragments in the intestine. By using a strain which produces a INF-gamma neutralising antibody fragment, inflammatory bowel disorder could be treated. Another aspect of the invention is a multivalent VHH comprising two or more VHHs each directed against IFN-gamma for use in the treatment, prevention and/or alleviation of disorders wherein the immune system is overactive, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is the use of a multivalent VHH comprising two or more VHHs each directed against INF-gamma for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders wherein the immune system is over active, wherein said VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders wherein the immune system is overactive, comprising administering to a subject a VHH specific for an antigen related to the disorder orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a multivalent VHH comprising two or more VHHs each directed against IFN-gamma for use in the preparation of a medicament for the treatment, prevention and/or alleviation of disorders wherein the immune system is

overactive. Another aspect of the invention is a use of a multivalent VHH comprising two or more VHHs directed against IFN-gamma for use in the preparation of a medicament for the treatment, prevention and/or alleviation of disorders wherein the immune system is over active. The anti-IFN-gamma VHHs of the present invention may be derived from the new class of VHHs described above, or may be derived from any of the other classes of VHHs, including the major class of VHH.

The above aspect and embodiments apply to a multivalent VHH comprising two or more anti-INF-gamma VHHs wherein said VHHs correspond to any from our future experiments, a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion.

Cloning of multivalent single-domain antibodies.

One embodiment of the present invention is a method of producing a multivalent single-domain antibody comprising the steps of sequential cloning of two or more gene segments coding for the different (or identical) single domain antibodies in an appropriate prokaryotic or eukaryotic expression system. The individual gene segments can be cloned by PCR, in which appropriate restriction sites are enforced within the FR1 and FR4 regions in such a way that problems with re-occurring sites can be avoided. In this application a method is described in which sites are used present within the vector originally used for cloning of the immune repertoires, thereby avoiding the PCR step, but leading to the incorporation of a short linker sequence consisting of part of the hinge and an Alanine repeat encoded by the NotI site. However, examples have been provided that the same multimeric molecules without such linker have identical binding characteristics.

Cloning procedures

The inventors designed an *E. coli* expression vector pAX11 which allowed a convenient two-step cloning of bivalent VHH as described in Example 18. The carboxy terminal VHH was cloned first with PstI and BstEII, while in the second step the other VHH is inserted by SfiI and NotI, which do not cut within the first gene fragment. The procedure avoids the enforcement of new sites by amplification and thus the risk of introducing PCR errors.

The inventors generated bivalent derivatives of VHH's , using this pAX11 vector. The plasmid vector encoding the bivalent VHH was used to generate a tri- and tetrameric derivative, which was accomplished by partial digestion of the plasmid with BstEII, which occurs in both VHH gene segments. The linearized vector was purified from gel, subsequently de-phosphorylated and used as acceptor for cloning of the BstEII fragment of approx. 350 bp that was obtained by complete digestion of the same plasmid. Ligation of the BstEII fragment alone prior to addition to the vector enhances the insertion of multimeric VHH encoding gene segments. After transformation in *E. coli* TG1 the resulting clones were screened by PCR with M13Rev and M13Fwd primers; since BstEII is an a-symmetric cutter (5 nt overhang) only correctly-oriented inserts were obtained as was confirmed by digesting the plasmids with PstI alone (350 bp) or double digesting with EcoRI and HindIII (1000 bp for bivalent, 1350 bp for trivalent and 1700 bp for tetravalent, data not shown).

This methods disclosed in Example 18 and in this application can be readily understood by the skilled artisan an applied to the production of multivalent VHHs.

One embodiment of the present invention is a DNA cloning vehicle (called pAX11) suitable for cloning and expressing multivalent single domain antibodies, which is based on the *E. coli* vector pHEN1 (Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, Winter G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res. 19(15):4133-4137). pHEN1 is derived from pUC119 and it allows the inducible expression driven by the lac promoter; transformants containing this plasmid construct can be selectively grown on ampicillin containing media. The major differences between pAX11 and pHEN1 are the use of different restriction sites in the multiple cloning site, which are compatible with the cloning of single-domain antibody genes, the addition of a hexahistidine and a MYC-tag for purification and detection purposes and finally the deletion of the gene of M13 gene3, which is necessary for phage display, but has a negative influence on expression levels.

By transforming a compatible host, the homodimeric construct can be produced in sufficient quantities for use in therapy. One of the organisms of choice is *E. coli* or the yeast *Saccharomyces cerivisiae* which has been shown in EP698097 to be capable of producing VHH's in large quantities.

One embodiment of a DNA encoding a multivalent VHH as disclosed herein. Another embodiment of the present invention is nucleic acid capable of encoding a multivalent VHH as disclosed herein for use in the treatment, prevention and/or alleviation of disorders as disclosed herein. Another embodiment of the present invention is the use of a nucleic acid capable of encoding a multivalent VHH as disclosed herein for use in the preparation of a medicament for the treatment, prevention and/or alleviation of disorders as disclosed herein.

In general, "therapeutically effective amount", "therapeutically effective dose" and "effective amount" means the amount needed to achieve the desired result or results. One of ordinary skill in the art will recognize that the potency and, therefore, an "effective amount" can vary for the various compounds used in the invention. One skilled in the art can readily assess the potency of the compound.

As used herein, the term "compound" refers to a multivalent VHH as disclosed herein, a homologous sequence thereof, or a homologue thereof, or a nucleic acid capable of encoding said polypeptide or an agent identified according to the screening method described herein or said polypeptide comprising one or more derivatised amino acids.

By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual along with the compound without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Multivalent VHHs as disclosed herein are useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound or composition.

Multivalent VHHs as disclosed herein useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound combination with another, such as, for example, aspirin.

The present invention is not limited to the administration of formulations comprising a single compound of the invention. It is within the scope of the invention to provide combination

treatments wherein a formulation is administered to a patient in need thereof that comprises more than one compound of the invention.

A compound useful in the present invention can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient or a domestic animal in a variety of forms adapted to the chosen route of administration, *i.e.*, orally or parenterally, by intranassally by inhalation, intravenous, intramuscular, topical or subcutaneous routes.

A compound of the present invention can also be administered using gene therapy methods of delivery. See, *e.g.*, U.S. Patent No. 5,399,346, which is incorporated by reference in its entirety. Using a gene therapy method of delivery, primary cells transfected with the gene for the compound of the present invention can additionally be transfected with tissue specific promoters to target specific organs, tissue, grafts, tumors, or cells.

Thus, the present compound may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to

materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compound may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Examples of useful dermatological compositions which can be used to deliver the compound to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

An administration regimen could include long-term, daily treatment. By "long-term" is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. See Remington's Pharmaceutical Sciences (Martin, E.W., ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

Kits

The invention also provides for kits useful for the diagnosis of disorders, said diseases characterised by one or more targets against which the VHHs of the multivalent VHH disclosed herein is directed to. Kits useful according to the invention can include an isolated antigen, such as, for example, TNF-alpha, vWF., IFN-gamma or any other target, or fragment thereof. Alternatively, or in addition, a kit can comprise cells transformed to express target, such as, for example, TNF-alpha, vWF., IFN-gamma or any other antigen, or fragment thereof. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding TNF-alpha, or fragment thereof. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding vWF, or fragment thereof.. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding IFN-gamma, or fragment thereof. In a still further embodiment, a kit according to the invention may comprise the specific primers useful for amplification of TNF-alpha, or fragment thereof, vWF, or fragment thereof, or IFN-gamma, or fragment thereof. Kits useful according to the invention can comprise a multivalent VHH or fragment thereof, directed against any of the aforementioned antigens. For example, the kit may comprise a multivalent-VHH comprising two or more VHHs directed against TNF-alpha; the VHHs may correspond to sequences represented by any of SEQ ID NOs: 18 to 32 other anti-TNF-alpha VHHs, a homologue thereof, or a functional portion thereof. In another example, the kit may comprise a multivalent-VHH comprising two or more VHHs directed against vWF; the VHHs may correspond to sequences represented by any of SEQ ID NOs: 1 to 16, other anti-vWF VHHs, a homologue thereof, or a functional portion thereof. In another example, the kit may comprise a multivalent-VHH comprising two or more VHHs directed against IFN-gamma; the VHHs may correspond to sequences represented by any resulting from our future experiments, other anti-IFN-gamma VHHs, a homologue thereof, or a functional portion thereof. A kit according to the invention can comprise cells transformed to express said polypeptide. Kits may contain more than one polypeptide. In a further embodiment, a kit according to the invention can comprise a polynucleotide encoding said multivalent VHHs, or fragment thereof. In a still further embodiment, a kit according to the invention may comprise the specific primers useful for amplification of a macromolecule such as, for example, TNF-alpha, vWF, IFN-gamma or any other target, or a fragment thereof. All kits according to the invention will comprise the stated items or combinations of items and packaging materials therefore. Kits will also include instructions for use.

A method for diagnosing a disorder characterised by the dysfunction of a target according to the invention comprising:

(a) contacting a sample with a multivalent VHH according to the invention,

(b) detecting binding of said multivalent VHH to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of the target.

Techniques for performing the above method are known to the skilled artisan.

FIGURES

Figure 1: Phage ELISA for vWF for VHH libraries of PBLs (first and second bleeding) versus lymph node punctions.

Figure 2: Binding in ELISA of purified AM-4-15-3 to vWF.

Figure 3: ELISA to test inhibition by AM-4-15-3 of binding of vWF to collagen.

Figure 4: Amino acid sequences of bivalent AM-4-15-3 (BV-AM-4-15-3).

Figure 5: Binding curves of monovalent and bivalent AM-4-15-3 to vWF.

Figure 6: Inhibition curves of vWF binding to collagen by monovalent and bivalent AM-4-15-3.

Figure 7: Stability of bivalent AM-4-15-3 in plasma.

Figure 8: Sequences of anti TNF-alpha antibodies.

Figure 9: Binding assay to TNF-alpha.

Figure 10: EcoRI – HindIII insert of vector pAX11 (pUC119 backbone) for production of bivalent or bispecific VHH.

Figure 11: Coomassie-stained PAGE (15%) of IMAC-purified mono-(lane 8), bi- (lane 1), tri- (lanes 2,3 and 5) and tetravalent (lanes 4, 6 and 7) anti-TNF-alpha VHH.

Figure 12: Chromatogram of the analysis by gel filtration on Superdex 75HR of the mono-, bi-, tri and tetravalent VHH.

Fig 13: Comparison of the antagonistic characteristics of the mono-, bi-, tri-, and tetravalent form of the anti-human TNF-alpha VHH with the clinically used products Remicade and Enbrel.

Figure 14: Antagonistic behaviour of the mono- and bivalent VHH's directed against mouse TNF-alpha.

Figure 15: Western blot for expression of the A1 and A3 domain of vWF on the surface of *E.coli* as a fusion with OprI.

Figure 16: The results of experiments to determine the inhibitory effect of VHH upon the binding of vWF to the platelet receptor gpIb as in Example 27.

NAME	SEQ ID NO	SEQUENCE
C37	1	QVQLQESGGGLVQPGGSLRLSCAASGFNFNWYPMSWVRQAPGKGLEW VSTISTYGEPRYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYYCAR GAGTSSYLPQRGNWDQGTQVTISS
T76	2	QVQLQESGGGLVQPGESLRLSCAASGSIFSINTMGWYGQAPGKQRELVA SITFGGVTNYADSVKGRFTISRDNTNDTVYLQMNSLKPEDTAVYICNAVTW GGLTNYWGQGTQVTVSS
Z29	3	QVQLQESGGGSVQAGDSLTLSCAASGRTFSMHAMGWFRQAPGKEREFV AAISPSAFTTEYADSLKGRFTVSRDNAKKLVWLQMNGLKPEDTAAYYCAA RRGAFTATTAPLYDYWGQGTQVTVSS
A50	4	QVQLQESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFV AAISRRGDNVYYADSVKGRFAISRDNAESTLYLQMNSLKPEDTAVYYCAA HVTVSAITLSTSTYDYWGQGTQVTVSS
A38	5	QVQLQDSGGGSVQAGGSLRLSCAASGRTVSSYNMGWFRRVPGKERDFV AAISWSGVATYYFDSVKGRFTISRDNAKNTVYLEMNSLKPEDTAVYYCAAA SRYRHRLNSGSEYDYWGQGTQVTVSS
I53	6	QVQLQDSGGGLVQAGGSLRLSCAASGRTKDMAWFRQPPGKEREFVAVIY SSDGSTLVAASVKGRFTISRDNAKNTVYLQMTSLKPADTAVYYCATSRGY SGTYYSTSRYDYWTGGTQVTVSS
M53	7	QVQLQDSGGGLVQAGESLRLSCGTSGRTFGRRAMAWFRQAPGKERQFV AWIARYDGSTLYADSVKGRFTISRDDNKNTMYLHMNNLTPEDTAVYYCAA GPRGLYYESRYEYWGQGTLVTVSS
22-2L-34	8	QVQLQDSGGGLVQAGGSLRLSCAASVRIFTSYAMGWFRQAPGKEREFVA AINRSGKSTYYSDSVEGRFTISRDNAKNTVSLQMDSLKLEDTAVYYCAA DYSGSYTSLWSRPERLDWGQGTQVTVFS
22-4L-16	9	QVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFV A AISWSGGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCVA DTGGISWIRTQGYNYWGQGTQVTVSS
AM-4-15-3	10	QVQLQDSGGGLVQPGGSLRLACAASGSIFSINSMGWYRQAPGKQRELVA HALAD GSASYRDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTK GYWG QGTQVTVSS

Table 1-1: Sequences of VHH directed against von Willebrand Factor

AM-2-75	11	QVQLQESGGGLVQPGGSLRLSCAASGFNFNWYPMSWVRQAPGKGLEW VSTISTYGEPRYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYYCAR GAGTSSYLPQRGNWDQGTQVTVSS
2A1-4L-79	12	QVQLQDSGGRLVKAGASLRLSCAASGRTFSSLPMAWFRQAPGKEREFVA FIGSDSSTLYTSSVRGRFTISRDNGKNTVYLQMMNLKPEDTAVYYCAARS SAFSSGIYYREGSYAYWGQGTQVTVSS
2A1-4L-129	13	QVQLQESGGGLVQAGASLRLSCAASGRSFSSYPMAWFRQAPGKEREFV VFIGSDHSTLYSTSVRGRFTISRDNAKNTVYLQMMNLKPEDTAVYYCAAR NSAWSSGIYYRETSYDYWGQGTQVTVSS
2A1-4L-34	14	QVQLQDSGGGSVQAGASLRLSCAASGGTFSSYAMAWFRQAPGKEREFV GFIGSDGSTLYSSSVRGRFTISRDNAKNTVALQMMNLKPEDTAVYYCAAR ARYSGIYYRETDYPYWGQGTQVTVSS
2A1-4L-78	15	QVQLQESGGGLVQAGASLRLSCTASGRSFGGFPMGWFRQAPGKEREFV SGLTRSLFTVYADSVKGRFTVSTDNTKNTVYLQMNSLKPEDTAVYYCAAR PDLYAYSRDPNEYDYWGQGTQVTVSS
2LA1-15	16	QVQLQDSGGGLVQSGGSLRLACAASGRIVSTYAMGWFRQSPGKEREFV ATVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCAKTKRTGIFTTARMVD YWGQGTQVTVSS

Table 1-1 condt.....: Sequences of VHH directed against von Willebrand Factor

BV-AM-4-15-3	17	QVQLQDSGGGLVQPGGSLRLACAASGSIFSINSMGWYRQAPGKQRELVAHALAD GSASYRD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTKGYWGQGTQVTV SSEPKTP KPQPAAAQVQLQDSGGGLVQPGGSLRLACAASGSIFSINSMGWYRQAPGKQREL VAHALAD GSASYRDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTKGYWG QGTQVTV SS

Table 1-2: Sequences of bivalent VHH directed against von Willebrand Factor

TABLE 1-2

NAME	SEQ ID NO	SEQUENCE
VHH#1	18	QVQLQESGGGLVQPGGSLRLSCATSGFDFSVSWMYWVRQAPGKGLEWVSEINTNG LITKYVDSVKGRFTISRDNAKNTLYLQMDSLIPEDTALYYCARSPSGSFRGQGTQ VTVSS
VHH#9	19	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITSGD NLNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNAILQTSRWSIPSNY WGQGTQVTVSS
VHH#13	20	QVQLQESGGGLVQPGGSLRLSCATSGFTFSDYWMYWVRQAPGKGLEWVSTVNTNG LITRYADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVVPPYSDDSRTN ADWGQGTQVTVSS
VHH#2	21	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVAR IYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTS RSVESYNYWGQGTQVTVSS
VHH#3	22	QVQLQDSGGGLVQAGGSLRLSCAVSGRTFSAHSVYTMGWFRQAPGKEREFVARIY WSSANTYYADSVKGRFTISRDNAKNTVDLLMNSLKPEDTAVYYCAARDGIPTSRT VGSYNYWGQGTQVTVSS
VHH#4	23	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITSSD TNDTTNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNAVLQTSRWSIP SNYWGQGTQVTVSS
VHH#5	24	QVQLQDSGGGLVQAGGSLRLSCTTSGRTISVYAMGWFRQAPGKEREFVASISGSG AITPYADSVKGRFTISRDNAKNTVYLQMNSLNPEDTAVYYCAASRYARYRDVHAY DYWGQGTQVTVSS
VHH#6	25	QVQLQDSGGGLVQAGGSLRLSCAASTRTFSRYVVGWFRQAPGKEREFVATISWNG EHTYYADSVKGRYTISRDNAKNTVYLQMGSLKPEDTAVYYCAARSFWGYNVEQRD FGSWGQGTPVTVSS
VHH#7	26	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITNDT TNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNTVLQTSRWNIPTNYW GQGTQVTVSS
VHH#8	27	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIISGDT TNYADAVKGRFTISTDNVKKTVYLQMNVLESEDTAVYYCNAVLQTSRWSIPSNYW GQGTQVTVSS
VHH#10	28	QVQLQDSGGGLVQPGGSLRLACVASGSIFSIDVMGWYRQAPGQQRELVATITNSW TTNYADSVKGRFTISRDNAKNVVYLQMNSLKLEDTAVYYCNARRWYQPEAWGQGT QVTVSS

VHH#11	29	QVQLQDSGGGLVQPGGSLRLSCAASGFTFSTHWMYWVRQAPGKGLEWVSTINTNG LITDYIHSVKGRFTISRDNAKNTLYLQMNSLKSEDTAVYYCALNQAGLSRGQGTQ VTVSS
VHH#12	30	QVQLQESGGGLVQAGGSLRLSCAASRRTFSGYAMGWFRQAPGKEREFVAVVSGTG TIAYYADSVKGRFTISRDNAENTVYLQMNSLKPEDTGLYYCAVGPSSSRWYYRGA SLVDYWGKGTLVTVSS
VHH 9E	31	EVQLVESGGGLVQAGGSLRLSCAASGGTLSSYITGWFRQAPGKEREFVGAVSWSS STIVYADSVEGRFTISRDNHQNTVYLQMDSLKPEDTAVYYCAARPYQKYNWASAS YNVWGQGTQVTVSS
VHH 3F	32	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAPGKEREFVGAVSWSG GTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYCAARPYQKYNWASAS YNVWGQGTQVTVSS

Table 1-3: Sequences of VHH directed against TNF-alpha

BIV 3F	33	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAPGKEREFVGAVSWSG GTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYCAARPYQKYNWASAS YNVWGQGTQVTVSSEPKTPKPQPAAAQVQLQDSGGGLVQAGGSLRLSCAASGGTF SSIIMAWFRQAPGKEREFVGAVSWSGGTTVYADSVLGRFEISRDSARKSVYLQMN SLKPEDTAVYYCAARPYQKYNWASASYNVWGQGTQVTVSS
BIV #2	34	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVAR IYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTS RSVESYNYWGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLSCAA SGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRFAISRDI AKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS
TRIV #2	35	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVAR IYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPTS RSVESYNYWGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPGGSLRLSCAA SGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGNTYYADSVKGRFAISRDI AKNTVDLTMNNLEPEDTAVYYCAARDGIPTSRSVESYNYWGQGTQVTVSSEPKTP KPQPAAAQVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGK EREFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAA RDGIPTSRSVESYNYWGQGTQVTVSS

Table 1-4: Sequences of bivalent (BIV 3F, BIV #2) or trivalent (TRIV #2) VHH directed against TNF-alpha

EXAMPLES

The invention is illustrated by the following non-limiting examples.

Example 1: Immunization of llamas

A llama was immunized with vWF (Von Willebrand Factor was purchased from the Red Cross (Belgium)). The immunization scheme is summarized in Table 2.

Day of immunization	vWF
0	100 µg
7	100 µg
14	50 µg
21	50 µg
28	50 µg
35	50 µg

Table 2: Immunization scheme used according to Example 1.

Example 2: Repertoire cloning

Different sources for RNA extraction were used:

- 150 ml immune blood, between 4 and 10 days after the last antigen injection
- lymph node biopsy 4 days after the last antigen injection

Peripheral blood lymphocytes (PBLs) were isolated by centrifugation on a density gradient (Ficoll-Paque Plus Amersham Biosciences). PBLs and lymph node were used to extract total RNA (Chomczynski and Sacchi 1987) followed by synthesis of cDNA using a hexanucleotide random primer. The repertoire was amplified using two hinge-specific primers: AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG and AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT and a framework 1 specific primer: GAGGTBCARCTGCAGGASTCYGG. Fragments were digested with PstI and NotI and cloned into a phagemid vector. The repertoire was transformed in TG1 electrocompetent cells and plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. Colonies were screened for the presence of insert by PCR with vector specific primers. Results are summarized in Table 3.

#days after last injection	Source RNA	Size of the library	% insert
4	Lymph	1.3×10^7	89
7	PBL	1.9×10^7	95
10	PBL	1.1×10^9	70

Table 3: Results of cloning of VHH repertoire in a phagemid vector with different sources of RNA and different time points after the last immunization.

Example 3: Rescue of the library, phage preparation

Libraries were grown at 37°C in 10 ml 2xTY medium containing 2% glucose, and 100 µg/ml ampicillin, until the OD600nm reached 0.5. M13KO7 phages (10^{12}) were added and the mixture was incubated at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. Cells were centrifuged for 10 minutes at 4500 rpm at room temperature. The bacterial pellet was resuspended in 50 ml of 2xTY medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated overnight at 37°C with vigorously shaking at 250 rpm. The overnight cultures were centrifuged for 15 minutes at 10.000 rpm at 4°C. Phages were PEG precipitated (20% poly-ethylene-glycol and 1.5 M NaCl) and centrifuged for 30 minutes at 10.000 rpm. The pellet was resuspended in 20 ml PBS. Phages were again PEG precipitated and centrifuged for 30 minutes at 20,000 rpm and 4°C. The pellet was resuspended in 5 ml PBS-1% casein. Phages were titrated by infection of TG1 cells at OD600nm= 0.5 and plating on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. The number of transformants indicates the number of phages (= pfu). The phages were stored at –80°C with 15% glycerol.

Example 4: Phage ELISA

A microtiter plate (Maxisorp) was coated overnight at 4°C with PBS-1% casein or with 2 µg/ml vWF. The plate was washed 3 times with PBS-Tween (0.05% Tween20) and blocked for 2 hours at room temperature with 200 µl PBS-1% casein. The plate was washed five times with PBS-Tween. Phages were prepared as described above and applied to the wells in duplo dilutions. Plates were washed five times with PBS-Tween. Binding phages were detected with a mouse anti-M13 conjugated with HRP diluted 1/2000 in PBS. The plates were washed five times with PBS-Tween. Staining was performed with ABTS/H2O2 and signals were measured after 30 minutes at 405 nm. The results are presented in Figure 1. It is clear from the results that specific VHH are present in all libraries.

Example 5: Selection for binders for vWF inhibiting the interaction with collagen

A well in a microtiterplate was coated with 2 µg/ml vWF or with PBS containing 1% casein. After overnight incubation at 4°C, the wells were blocked with PBS containing 1% casein, for 3 hours at RT. 200 µl phages were pooled for the three libraries and 100 µl of this pool was added to the wells. After 2 hours incubation at RT, the wells were washed 10x with PBS-Tween and 10x with PBS. Phages were specifically eluted with 100 µl of 100 µg/ml collagen type III (human placental collagen type III was purchased from Sigma (St. Louis, MO); collagen was solubilized in 50 mM acetic acid at 5mg/ml and diluted with PBS to 1mg/ml to obtain fibrillar collagen). Elutions were performed overnight at room temperature. Eluted phages were allowed to infect exponentially growing TG1 cells, and were then plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. The results from the panning experiment are summarized in Table 4.

Pfu vWF	Pfu casein	Enrichment
1×10^7	2.5×10^5	40

Table 4: Plaque forming untis (pfu) after one round of panning on vWF as compared to PBS-casein. Pfu vWF (antigen) divided by pfu casein (aspecific binding) = enrichment.

Example 6: Functional characterization of vWF binders: Inhibition of binding of vWF to collagen by VHH

A microtiter plate was coated overnight at 4°C with collagen type III at 25 µg/ml in PBS. The plate was washed five times with PBS-tween and blocked for 2 hours at room temperature with PBS containing 1% casein. The plate was washed five times with PBS-tween. 100 µl of 2 µg/ml vWF (vWF was pre-incubated at 37°C for 15 minutes) was mixed with 20 µl periplasmic extract (prepared according to example 7 but in TG1 cells) containing a VHH antibody for testing and incubated for 90 minutes at room temperature in the wells of the microtiterplate. The plate was washed five times with PBS-tween. An anti-vWF-HRP monoclonal antibody (HRP anti-M13 monoclonal conjugate was purchased from Amersham Biosciences) was diluted 3,000-fold in PBS and incubated for 1 hour. The plate was washed five times with PBS-tween and vWF-binding was detected with ABTS/H2O2. Signals were measured after 30 minutes at 405 nm. 600 individual colonies were tested and 4 clones inhibited the interaction between collagen and vWF.

Example 7: Expression and purification of one VHH: AM-4-15-3

Plasmid was prepared for one binder (AM-4-15-3) for vWF inhibiting the interaction with collagen typeIII and was transformed into WK6 electrocompetent cells. A single colony was used to start an overnight culture in LB containing 2% glucose and 100 µg/ml ampicillin. This overnight culture was diluted 100-fold in 300 µl TB medium containing 100 µg/ml ampicillin, and incubated at 37°C until OD600nm= 0.5. 1 mM IPTG was added and the culture was incubated for 3 more hours at 37°C. Cultures were centrifuged for 20 minutes at 10.000 rpm at 4°C. The pellet was froozen overnight or for 1 hour at -20°C. Next, the pellet was thawed at room temperature for 40 minutes, re-suspended in 20 ml PBS and shaken on ice for 1 hour. Periplasmic fraction was isolated by centrifugation for 20 minutes at 4°C at 20.000 rpm. The supernatant containing the VHH was loaded on Ni-NTA and purified to homogeneity.

Example 8: ELISA: binding to vWF

A microtiter plate was coated with 2 µg/ml vWF, overnight at 4°C. Plates were blocked for two hours at room temperature with 300 µl 1% casein in PBS. The plates were washed three times with PBS-Tween. Dilution series (67 nM to 7 pM, dilution factor three) of the purified sample of AM-4-15-3 was incubated for 2 hours at RT. Plates were washed six times with PBS-Tween, after which binding of VHH was detected by incubation with mouse anti-myc mAB 1/2000 in PBS for 1 hour at RT followed by anti-mouse-HRP conjugate 1/1000 in PBS, also for 1 hour at RT. Staining was performed with the substrate ABTS/H2O2 and the signals were measured after 30 minutes at 405 nm. The binding as a function of concentration of purified AM-4-15-3 is indicated in Figure 2.

Example 9: specificity of AM-4-15-3

Microtiterplates were coated with 2 µg/ml vWF and 3 other antigens not involved in platelet aggregation, but that were also used for immunization in the same animal. ELISA was performed as described in example 8 with 670, 67 and 6.7 nM VHH. Results are summarized in table 5.

OD405 nm	vWF			Antigen 1			Antigen 2			Antigen 3		
nM	670	67	6.7	670	67	6.7	670	67	6.7	670	67	6.7
AM-4-15-3	1.32	1.06	0.56	0.09	0.12	0.12	0.12	0.11	0.10	0.10	0.10	0.08

Table 5: OD 405 nm for binding of AM-4-15-3 to vWF and 3 antigens that were also immunized in the same animal.

Example 10: inhibition ELISA with purified VHH

Inhibition ELISA was performed as described in example 6 but with different concentrations of AM-4-15-3. Instead of pure vWF, human plasma was used at a 1/60 dilution (final concentration). Results are represented in figure 3. The concentration of VHH resulting in 50% inhibition (IC50) is 2 nM.

Example 11: Sequencing of AM-4-15-3

AM-4-15-3 was sequenced with M13 universal reverse primer.

AM-4-15-3

QVQLQDSGGGLVQPGGSLRLACAASGSIFSINSMGWYRQAPGKQRELVAHALADGSASYRDSVKGR
FTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTKGYWGQGTQVTVSS

Example 12: Construction of bivalent AM-4-15-3

Bivalent AM-4-15-3 was prepared as described (Conrath et al, JBC 10, 7346-7350). We used the middle hinge of the llama as a linker between both VHH's. The sequence is shown in figure 4 (bold= hinge).

Example 13: expression and purification of bivalent AM-4-15-3

Protein was expressed and purified as described in example 7. A extra purification step was needed on Superdex 75 for removal of some monovalent degradation product (5-10%). We obtained 2.3 mg of purified bivalent protein per 1 liter *E.coli* culture .

Example 14: binding to vWF for monovalent versus bivalent AM-4-15-3

Binding to vWF was tested in ELISA as described in example 8 and compared to binding of monovalent AM-4-15-3. The results are shown in Figure 5.

Example 15: Inhibition of binding of vWF to collagen with monovalent versus bivalent AM-4-15-3

Inhibition for binding of vWF to collagen was tested for monovalent as compared to bivalent constructs as described in example 6. Instead of using vWF, human plasma was used at a dilution of 1/60. IC50 value for monomer is 2 nM as compared to 20 pM for the bivalent construct. This is an improvement by a factor of 100. The results are shown in Figure 6.

Example 16: Stability of bivalent AM-4-15-3 in human plasma

Stability of the bivalent construct was tested by incubation at 37°C in human plasma at a concentration of 38 µg/ml. A sample was removed after 1, 2, 3, 6 and 24 hours incubation. Samples were diluted 10-fold and analyzed by Western blot. Results are summarized in figure 7 and show that the bivalent construct is stable for 24 hours at 37°C in human plasma.

Example 17: Cloning and characterization of anti-TNFα antibodies

1) Immunization and library constructions

A llama (*Llama glama*) was immunized with human TNF-alpha. For immunization, the cytokine was formulated as an emulsion with an appropriate, animal-friendly adjuvant (Specoll, CEDI Diagnostics B.V.). The antigen cocktail was administered by double-spot injections intramuscularly in the neck. The animal received 6 injections of the emulsion, containing 100 µg of TNF-α at weekly intervals. At different time points during immunization, 10-ml blood samples were collected from the animal and sera were prepared. The induction of an antigen specific humoral immune response was verified using the serum samples in an ELISA experiment with TNF (data not shown). Five days after the last immunization, a blood sample of 150 ml was collected. From this sample, conventional and heavy-chain antibodies (HcAbs) were fractionated (Lauwereys et al. 1998) and used in an ELISA, which revealed that the HcAbs were responsible for the antigen specific humoral immune response (data not shown). Peripheral blood lymphocytes (PBLs), as the genetic source of the llama heavy chain immunoglobulins (HcAbs), were isolated from the 150-ml blood sample using a Ficoll-Paque gradient (Amersham Biosciences) yielding $5x10^8$ PBLs. The maximal diversity of antibodies is expected to be equal to the number of sampled B-lymphocytes, which is about 10 % of the number of PBLs ($5x10^7$). The fraction of heavy-chain antibodies in llama is up to 20 % of the number of B-lymphocytes. Therefore, the maximal diversity of HcAbs in the 150 ml blood sample is calculated as 10^7 different molecules. Total RNA (around 400 □g) was isolated

from these cells using an acid guanidinium thiocyanate extraction (Chomczynski and Sacchi, 1987).

cDNA was prepared on 100 □g total RNA with M-MLV Reverse Transcriptase (Gibco BRL) and oligo-dT-primer or hexanucleotide random primers (Amersham Biosciences) as described before (de Haard *et al.*, 1999). The cDNA was purified with a phenol/chloroform extraction combined with an ethanol precipitation and subsequently used as template to specifically amplify the VHH repertoire.

The VHH repertoire was amplified using oligo-dT primed cDNA as template with a single degenerated framework1 (FR1) primer ABL013 (5'-GAGGTBCAR**CTGCAG**GASTCYGG-3'), introducing a *Pst*I restriction site (in bold), in combination with the oligo-dT primer as is described in EP01205100.9. This amplification yields two fragments of 1650 bp and 1300 bp, the latter being the product derived from the CH1-deleted HcAb genes. The smaller PCR-product was gel purified and subsequently digested with *Pst*I and *Bst*EII. The *Bst*EII-site frequently occurs within the FR4 of heavy-chain derived VHH encoding DNA-fragments. Alternatively, the VHH-repertoire was amplified in a hinge-dependent approach using two IgG specific oligonucleotide primers. In a single PCR reaction a short (5'-AACAGTTAAGCTTCCGCTT**GCGGCCGC**GGAGCTGGGGTCTTCGCTGTGGTGCG-3') or long (5'-AACAGTTAAGCTTCCGCTT**GCGGCCGC**TGGTTGTGGTTTTGGTGTCTTGGGTT-3') hinge primer known to be specific for HcAbs was combined with the FR1-primer ABL013 (see above). A *Pst*I and *Not*I (bold underlined) restriction site was introduced within the FR1 and hinge primers respectively, to allow cloning. Subsequently, the DNA fragments were ligated into *Pst*I-*Bst*EII or *Pst*I-*Not*I digested phagemid vector pAX004, which is identical to pHEN1 (Hoogenboom *et al.*, 1991), but encodes a carboxyterminal $(His)_6$- and c-myc-tag for purification and detection, respectively. The ligation mixture was desalted on a Microcon filter (YM-50, Millipore) and electroporated into *E. coli* TG1 cells to obtain a library containing $1.8x10^7$ clones. The transformed cells were grown overnight at 37°C on a single 20x20 cm plate with LB containing 100 μg/ml ampicillin and 2 % glucose. The colonies were scraped from plates using 2xTY medium and stored at -80°C in 20 % glycerol.

As quality control the percentage of insert containing clones was verified on 24 clones for each library by PCR using a combination of vector based primers. This analysis revealed that

95 % of the clones contained a VHH encoding insert. The variability was examined by *Hin*FI fingerprint analysis of the amplified VHH fragment of these 24 clones, thereby showing that all clones were indeed different (data not shown).

2) Selection of antagonistic anti-TNF VHH's

From both libraries phage was prepared. To rescue the polyclonal phage repertoire, libraries were grown to logarithmic phase (OD600 = 0.5) at 37°C in 2xTY containing 100 µg/ml ampicillin and 2 % glucose and subsequently superinfected with M13K07 helper phage for 30 minutes at 37°C. Infected cells were pelleted for 5 minutes at 4000 rpm and resuspended in 2xTY containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. Bacteriophage was propagated by overnight growth at 37°C and 250 rpm. Overnight cultures were centrifuged for 15 minutes at 4500 rpm and phage was precipitated with one fifth volume of a [20% polyethyleneglycol 6000, 1.5 M NaCl]-solution by incubation for 30 minutes on ice. Phage was pelleted by centrifugation for 15 minutes at 4000xg and 4°C. After resuspension of the phages in PBS, cell debris was pelleted by centrifugation for 1 minute at maximal speed (15000xg) in microcentrifuge tubes. The supernatant containing the phage particles was transferred to a new tube and again phage was precipitated as described above. Phage was dissolved in PBS and separated from remaining cell debris as mentioned above. The titer of phage was determined by infection of logarithmic TG1 cells followed by plating on selective medium.

The library was selected using *in vitro* biotinylated TNF-alpha. The biotinylation was carried out as described by Magni et al (Anal Biochem 2001, 298, 181-188). The incorporation of biotin in TNF was evaluated by SDS-PAGE analysis and detection with Extravidin-alkaline phosphatase conjugate (Sigma). The functionality of the modified protein was evaluated for its ability to bind to the solid phase coated recombinant a p75 receptor.

VHH were selected by capturing biotinylated TNF-alpha (10 to 400 ng per well during 2 hours at room temperature) on streptavidin coated microtiter plates (coated with 100 µl of 10 µg/ml streptavidin during 16 hours at +4°C). Antagonistic VHH were obtained by elution with an excess of receptor, either the extracellular ligand binding domain or with cells expressing the receptor. After 2 hours incubation of phage with captured cytokine, the non-specific phage was washed away, while specific phage displaying antagonistic VHH was eluted for 30

minutes with receptor (extracellular domain of CD120b or p75; 10 µM) or with receptor displaying cells (>10^7 KYM cells per well). High enrichments, i.e. the ratio of the number of phage eluted with receptor and those eluted by serum albumin (50 µg per well), of more than a factor of 20 suggested the successful selection of TNF specific clones. Alternatively, instead of elution with receptor a standard procedure was applied, in which a low pH causes the denaturation of VHH and / or antigen (0.1 M glycine buffer pH 2.5). Log phase growing *E. coli* cells were infected with the eluted and neutralized phage and plated on selective medium.

Individual clones were picked and grown in microtiter plate for the production of VHH in culture supernatant. ELISA screening with TNF captured on Extravidin coated plates revealed about 50% positive clones. *Hin*FI-fingerprint analysis showed that 13 different clones were selected, which were grown and induced on 50 ml scale. The sequences of said clones are shown in Table 6 below:

NAME	SEQ ID NO	SEQUENCE
VHH#1	11	QVQLQESGGGLVQPGGSLRLSCATSGFDFSVSWMYWVRQAPGKGLEWVSEIN TNGLITKYVDSVKGRFTISRDNAKNTLYLQMDSLIPEDTALYYCARSPSGSFRGQ GTQVTVSS
VHH#9	12	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITS GDNLNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNAILQTSRWSIPS NYWGQGTQVTVSS
VHH#13	13	QVQLQESGGGLVQPGGSLRLSCATSGFTFSDYWMYWVRQAPGKGLEWVSTV NTNGLITRYADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVYYCTKVVPPYSDD SRTNADWGQGTQVTVSS
VHH#2	14	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREF VARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGI PTSRSVESYNYWGQGTQVTVSS
VHH#3	15	QVQLQDSGGGLVQAGGSLRLSCAVSGRTFSAHSVYTMGWFRQAPGKEREFVA RIYWSSANTYYADSVKGRFTISRDNAKNTVDLLMNSLKPEDTAVYYCAARDGIPT SRTVGSYNYWGQGTQVTVSS
VHH#4	16	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITS SDTNDTTNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNAVLQTSRW SIPSNYWGQGTQVTVSS
VHH#5	17	QVQLQDSGGGLVQAGGSLRLSCTTSGRTISVYAMGWFRQAPGKEREFVASISG SGAITPYADSVKGRFTISRDNAKNTVYLQMNSLNPEDTAVYYCAASRYARYRDV HAYDYWGQGTQVTVSS
VHH#6	18	QVQLQDSGGGLVQAGGSLRLSCAASTRTFSRYVVGWFRQAPGKEREFVATIS WNGEHTYYADSVKGRYTISRDNAKNTVYLQMGSLKPEDTAVYYCAARSFWGY NVEQRDFGSWGQGTPVTVSS
VHH#7	19	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIITN DTTNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNTVLQTSRWNIPT NYWGQGTQVTVSS
VHH#8	20	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFVAIISG DTTNYADAVKGRFTISTDNVKKTVYLQMNVLESEDTAVYYCNAVLQTSRWSIPS NYWGQGTQVTVSS
VHH#10	21	QVQLQDSGGGLVQPGGSLRLACVASGSIFSIDVMGWYRQAPGQQRELVATITN

		SWTTNYADSVKGRFTISRDNAKNVVYLQMNSLKLEDTAVYYCNARRWYQPEA WGQGTQVTVSS
VHH#11	22	QVQLQDSGGGLVQPGGSLRLSCAASGFTFSTHWMYWVRQAPGKGLEWVSTIN TNGLITDYIHSVKGRFTISRDNAKNTLYLQMNSLKSEDTAVYYCALNQAGLSRGQ GTQVTVSS
VHH#12	23	QVQLQESGGGLVQAGGSLRLSCAASRRTFSGYAMGWFRQAPGKEREFVAVVS GTGTIAYYADSVKGRFTISRDNAENTVYLQMNSLKPEDTGLYYCAVGPSSSRWY YRGASLVDYWGKGTLVTVSS

Table 6: The sequences of said clones as obtained in Example 17.

Five clones, coded VHH#1, #2, #3, #9 and #13, with different sequences (Figure 8) were characterized in more detail :

Larger amounts of antibody fragments were expressed by cultivation on 50 ml scale and purified by IMAC using TALON resin (Clontech). After dialysis against PBS to remove the eluent imidazol the amount of VHH was determined by OD280; approximately 300 µg of VHH was obtained from each clone.

This material was used for determining the sensitivity of detection of (biotinylated) TNF in ELISA. For this purpose a streptavidin (10 µg/ml) coated microtiterplate was employed for capture of biotinylated VHH (1 µg/ml), VHH was diluted in 0.2 % casein / PBS and incubated for 2 hours at room temperature. Bound VHH was detected with anti-MYC mAB 9E10 (0.5 µg/ml) and anti-mouse AP conjugate (1000-fold diluted, Sigma). The results are shown in Figure 9.

Example 18: Increased antagonistic effect of multivalent anti-TNFα VHH

The *E. coli* production vector pAX11 (Figure 10) was designed, which allows the two-step cloning of bivalent or bispecific VHH. The carboxy terminal VHH is cloned first with PstI and BstEII, while in the second step the other VHH is inserted by SfiI and NotI, which do not cut within the first gene fragment. The procedure avoids the enforcement of new sites by amplification and thus the risk of introducing PCR errors.

With this vector the bivalent derivative of the antagonistic anti- human TNF-alpha VHH #2 was generated. The plasmid vector encoding the bivalent VHH was used to generate a tri- and tetrameric derivative, which was accomplished by partial digestion of the plasmid with BstEII, which occurs in both VHH gene segments. The linearized vector was purified from gel, subsequently de-phosphorylated and used as acceptor for cloning of the BstEII fragment of approx. 350 bp that was obtained by complete digestion of the same plasmid. Ligation of the BstEII fragment alone prior to addition to the vector enhances the insertion of multimeric VHH encoding gene segments. After transformation in *E. coli* TG1 the resulting clones were screened by PCR with M13Rev and M13Fwd primers; since BstEII is an a-symmetric cutter (5 nt overhang) only correctly oriented inserts were obtained as was confirmed by digesting the plasmids with PstI alone (350 bp) or double digesting with EcoRI and HindIII (1000 bp for bivalent, 1350 bp for trivalent and 1700 bp for tetravalent, data not shown).

The clones were grown and induced on 50 ml scale, periplasmic fractions prepared and used for IMAC purification with TALON resin. Analysis of the purified products on Coomassie stained PAGE revealed good production levels (between 2 and 10 mg per liter cell culture) of intact multivalent VHH (see Figure 11). The molecular appearance of the IMAC purified VHH was determined by gel filtration on a Superdex 75HR column and as expected the molecules with higher avidities came earlier from the column (see Figure 12).

The antagonistic efficacy was analyzed with a cell based assay, in which TNF-alpha-induced cytostasis/cytotoxicity was determined by the calorimetric MTT assay as described by Vandenabeele and colleagues (Vandenabeele, P., Declercq, W., Vercammen, D., Van de Craen, M., Grooten, J., Loetscher, H., Brockhaus, M., Lesslauer, W., Fiers, W. (1992) Functional characterization of the human tumor necrosis factor receptor p75 in a transfected rat/mouse T cell hybridoma. J. Exp. Med. 176, 1015-1024.). MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a pale yellow substrate that is cleaved by mitochondrial enzymes of living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. KYM cells (Sekiguchi M, Shiroko Y, Suzuki T, Imada M, Miyahara M, Fujii G. (1985) Characterization of a human rhabdomyosarcoma cell strain in tissue culture. Biomed. Pharmacother. 39, 372-380.) were seeded in 96 well microtiterplates and cultured in the presence or absence of TNF-alpha. (1.29 ng/ml or approx. 25 pM of trimer). In addition to TNF variable amounts of antibody (VHH or Remicade or its derived Fab) were included during cultivation. For the assay MTT was added to the culture medium at a final concentration of 500 ug/ml and the plates were incubated at 37°C to achieve cleavage of MTT by mitochondrial enzymes. The formed formazon product, which appear as black, fuzzy crystals on the bottom of the well are dissolved by addition of acid isopropanol (40 nM HCl in isopropanol) or DMSO. The absorbance is measured at 570 nm.

The MTT assays (Figure 13) revealed that the monovalent molecules used in this study had the poorest antagonistic characteristics, what is reflected by their IC50 values: the Fab derived from the chimeric antibody Remicade has an IC50 of 2 nM (not shown) and for VHH#2 it is 12 nM. The avidity of the used molecules turned out to have a dramatic influence on the antagonistic efficacy as was observed with the bivalent IgG molecule Remicade, which is 40-fold more effective (IC50 50 pM) than the Fab. TNF-alpha is a trimeric molecule, which

interacts to a dimeric receptor and therefore it can be expected that the avidity of the IgG permits the mutual binding to two epitopes on the cytokine and supports the formation of large complexes as has been described before (Santora et al, Anal.Biochem. 299, 119-129). Surprisingly, increasing the avidity of the VHH from monomer to dimer has a far more spectacular effect than observed with Remicade, since the IC50 of the dimer (30 pM) is 400 fold lower than of the monomer. Increasing the avidity even more leads to a still better antagonistic behaviour: the trimeric VHH has an IC50 of 20 pM and the tetravalent format 6 pM. All higher avidity formats of the VHH are more efficient than Remicade, while the tetravalent format is even better than Enbrel, which consists of the extracellular domain of the receptor p75 fused to the Fc of an IgG and therefore has a bivalent binding mode.

The same unexpected effect of avidity on antagonistic behaviour was observed with VHH generated against mouse TNF (Figure 14). The same type of cytotoxicity assay was performed using MTT as substrate and mouse TNF-alpha (65 pg/ml or 1.3 pM), but with the murine cell line L929, which expresses the mouse specific receptor. Three different antagonistic (monovalent) VHH were identified coded 9E and 3F, of which the first two have IC50's of 25 nM and the latter 2 nM. Conversion of 3F into the bivalent format yielded a 1000 fold increase in IC50 (2 pM), thereby demonstrating once more that the increased avidity of the antibody leads to an unexpected improvement of the antagonistic characteristics.

CLAIMS

1. A *Camelidae* multivalent VHH comprising two or more *Camelidae* VHHs directed against the same target.

2. A multivalent VHH according to claim 1 wherein said target is Tumour Necrosis Factor-alpha.

3. A multivalent VHH according to claim 1 wherein said two or more VHH correspond to sequences represented by any of SEQ ID NOs: 18 to 32.

4. A multivalent VHH according to claim 2 corresponding to the sequence represented by SEQ ID NO: 33 to 35.

5. A multivalent VHH according to any of claims 2 to 4, wherein said multivalent VHH is a homologous sequence of said multivalent VHH, a functional portion thereof, of an homologous sequence of a functional portion thereof.

6. A nucleic acid encoding a multivalent VHH according to any of claims 2 to 5.

7. A multivalent VHH according to any of claims 2 to 5, or a nucleic acid according to claim 6 for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

8. Use of a multivalent VHH according to any of claims 2 to 5, or a nucleic acid according to claim 6 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

9. A multivalent VHH of nucleic acid according to claim 7 or a use of a multivalent VHH according to claim 8 wherein said disorders are any of rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

10. A multivalent VHH or nucleic acid according to claims 7 and 9 or a use of a multivalent VHH according to claim 8 and 9 wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

11. A multivalent VHH according to claim 1 wherein said target is vWF .

12. A multivalent VHH according to claim 1 wherein said target is collagen.

13. A multivalent VHH according to claim 11 wherein said two or more VHH correspond to sequences represented by any of SEQ ID NOs: 1 to 16.

14. A multivalent VHH according to claim 11 corresponding to the sequence represented by SEQ ID NO: 17.

15. A multivalent VHH according to any of claims 11 to 14, wherein said multivalent VHH is a homologous sequence of said multivalent VHH, a functional portion thereof, of an homologous sequence of a functional portion thereof.

16. A nucleic acid encoding a multivalent VHH according to any of claims 11 to 15.

17. A multivalent VHH according to any of claims 11 to 15, or a nucleic acid according to claim 16 for use in the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof.

18. Use of a multivalent VHH according to any of claims 10 to 15, or a nucleic acid according to claim 16 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof.

19. A multivalent VHH or nucleic acid according to claim 17 or a use of a multivalent VHH or nucleic acid according to claim 18 wherein said disorders are any of cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, and said conditions are those arising from coronary by-pass

graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

20. A multivalent VHH or nucleic acid according to claims 17 and 19 or a use of a multivalent VHH according to claim 18 and 19 wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

21. A multivalent VHH according to claim 1 wherein said target is Interferon-gamma.

22. A multivalent VHH according to claim 21, wherein said multivalent VHH is a homologous sequence of said multivalent VHH, a functional portion thereof, of an homologous sequence of a functional portion thereof.

23. A nucleic acid encoding a multivalent VHH according to claims 21 and 22.

24. A multivalent VHH according to claim 21, or a nucleic acid according to claim 23 for use in the treatment, prevention and/or alleviation of disorders wherein the immune system is over-active.

25. Use of a multivalent VHH according to claims 21 and 22, or a nucleic acid according to claim 23 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders wherein the immune system is over-active.

26. A multivalent VHH or nucleic acid according to claim 24 or a use of a multivalent VHH according to claim 25 wherein said disorders are any of Crohn's disease, autoimmune disorders and organ plant rejection in addition inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

27. A multivalent VHH or nucleic acid according to claims 24 and 26 or a use of a multivalent VHH according to claim 25 and 26 wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

28. A composition comprising a multivalent VHH according to any of claims 1 to 5, 7, 9, 10, or a nucleic acid encoding said multivalent VHH and a pharmaceutically acceptable vehicle.

29. A composition comprising a multivalent VHH according to any of claims 11 to 15, 17, 19, 20, or a nucleic acid encoding said multivalent VHH and a pharmaceutically acceptable vehicle.

30. A composition comprising a multivalent VHH according to any of claims 22, 24, and 26, 27, or a nucleic acid encoding said multivalent VHH and a pharmaceutically acceptable vehicle.

31. A composition comprising a multivalent VHH according to claim 1, or a nucleic acid encoding said multivalent VHH and a pharmaceutically acceptable vehicle.

32. A method of diagnosing a disorder characterised by the dysfunction of Tumor Necrosis Factor-alpha comprising:

(a) contacting a sample with a multivalent VHH according to any of claims 2 to 5,

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of Tumor Necrosis Factor-alpha.

33. A kit for screening for a disorder characterised by the dysfunction of Tumor Necrosis Factor-alpha, said kit comprising a multivalent VHH according to any of claims 2 to 5 and a standard.

34. A method according to claim 32 or kit according to claim 33 wherein said disorders are any of rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

35. Use of a polypeptide according to any of claims 2 to 5 for the purification of said Tumor Necrosis Factor-alpha.

36. A method of diagnosing a disorder characterised by the dysfunction of von Willebrand Factor comprising:

(a) contacting a sample with a multivalent VHH according to any of claims 11 to 16,

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of von Willebrand Factor.

37. A kit for screening for a disorder characterised by the dysfunction of von Willebrand Factor, said kit comprising a multivalent VHH according to any of claims 11 to 16 and a standard.

38. A method according to claim 36 or kit according to claim 37 wherein said disorders are any of cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, and those arising from coronary by-pass graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

39. Use of a polypeptide according to any of claims 11 to 16 for the purification of said von Willebrand Factor.

40. A method of diagnosing a disorder characterised by the dysfunction of Interferon-gamma comprising:

(a) contacting a sample with a multivalent VHH according to claims 21 and 22,

(b) detecting binding of said polypeptide to said sample, and

(c) comparing the binding detected in step (b) with a standard, wherein a difference in binding relative to said sample is diagnostic of a disorder characterised by dysfunction of Interferon-gamma.

41. A kit for screening for a disorder characterised by the dysfunction of Interferon-gamma, said kit comprising a multivalent VHH according to claims 21 and 22 and a standard.

42. A method according to claim 40 or kit according to claim 41 wherein said disorders are any of Crohn's disease, autoimmune disorders and organ plant rejection in addition inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

43. Use of a polypeptide according to claims 21 and 22 for the purification of said Interferon-gamma.

44. A multivalent VHH according to claim 1 wherein said target is involved in a disease process.

45. A multivalent VHH according to claim 44, wherein said multivalent VHH is a homologous sequence of said multivalent VHH, a functional portion thereof, of an homologous sequence of a functional portion thereof.

46. A nucleic acid encoding a multivalent VHH according to claims 44 and 45.

47. A multivalent VHH according to claims 44 and 45, or a nucleic acid according to claim 46 for use in the treatment, prevention and/or alleviation of disorders or conditions in which the target is involved.

48. Use of a multivalent VHH according to any of claims 44 and 45, or a nucleic acid according to claim 45 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions in which the target is involved.

49. A multivalent VHH or nucleic acid according to claim 47 or a use of a multivalent VHH according to claim 48 wherein said multivalent VHH is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

50. A method of producing a polypeptide according to any of claims 1 to 5, 11 to 15, 21 and 22, 44 and 45 comprising

(a) culturing host cells comprising nucleic acid capable of encoding a polypeptide according to any of claims 1 to 5, 11 to 15, 21 and 22, 44 and 45, under conditions allowing the expression of the polypeptide, and,
(b) recovering the produced polypeptide from the culture.

51. A method according to claim 50, wherein said host cells are bacterial or yeast.

52. A multivalent single domain antibody comprising two or more a heavy chain immunoglobulin variable domains.

53. A multivalent antibody according to claim 52 comprising two or more variable domains directed against the same target.

54. A multivalent antibody according to claim 53 comprising two or more variable domains directed against the same or different epitopes on the same target.

ABSTRACT

The invention provides a multivalent ligand comprising heavy chain immunoglobulin variable domains which are directed to one or more functional antigen binding sites on the same antigen. The invention further relates to the said therapeutic molecules, compositions, uses, genetic constructions and methods of production.

FIGURE 1

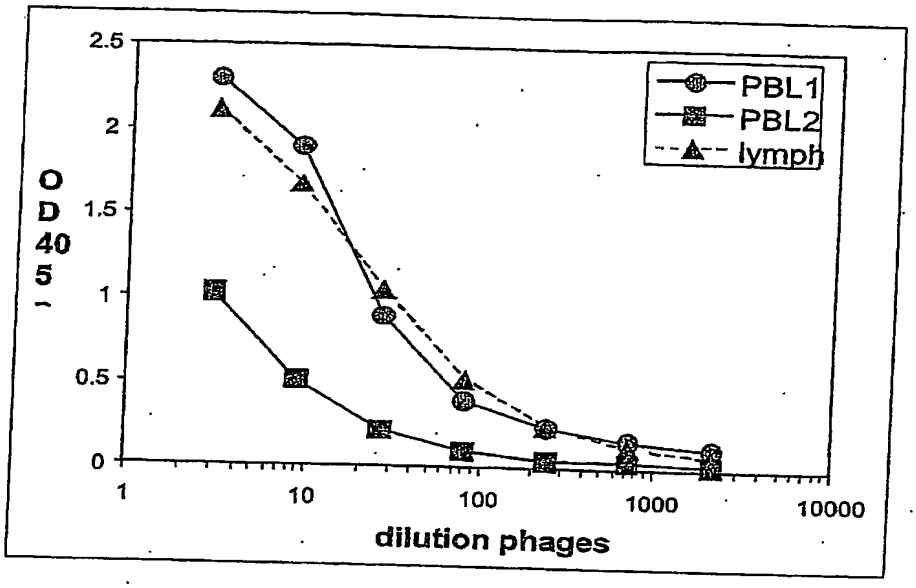

2.5
2
1.5
1
0.5
0
OD 405
1
10
100
1000
10000
dilution phages
PBL1
PBL2
lymph

FIGURE 2

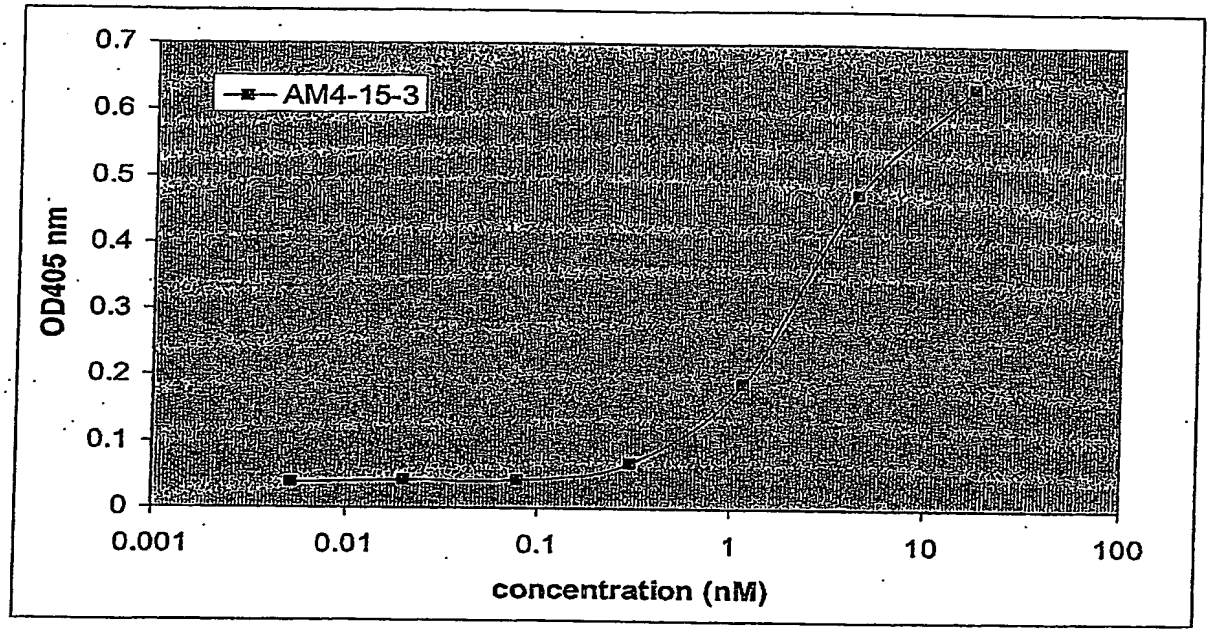

AM4-15-3
OD405 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
100
concentration (nM)

FIGURE 3

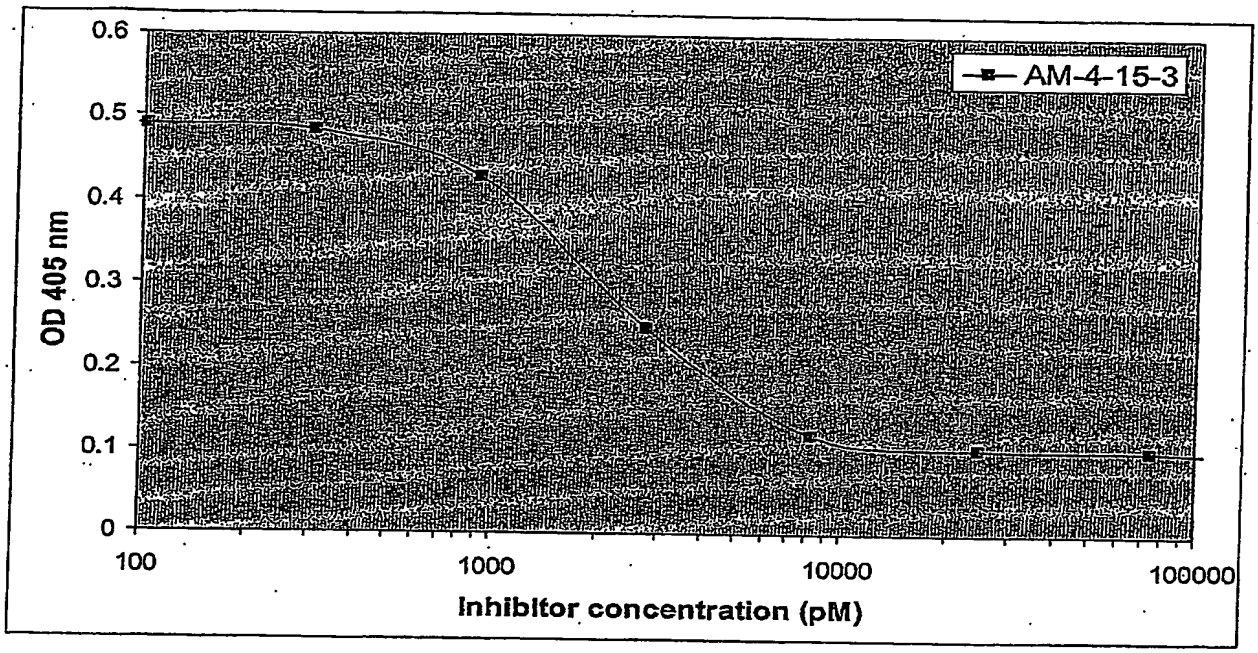

0.6
0.5
0.4
0.3
0.2
0.1
0
OD 405 nm
AM-4-15-3
100
1000
10000
100000
Inhibitor concentration (pM)

FIGURE 4

QVQLQDSGGGLVQPGGSLRLACAASGSIFSINSMGWYRQAPGKQRELVAHALADGSASYRDSVKG
RFTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTKGYWGQGTQVTVSSEPKTPKPQPAAAQ
VQLQDSGGGLVQPGGSLRLACAASGSIFSINSMGWYRQAPGKQRELVAHALADGSASYRDSVKGR
FTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTKGYWGQGTQVTVSS

FIGURE 5

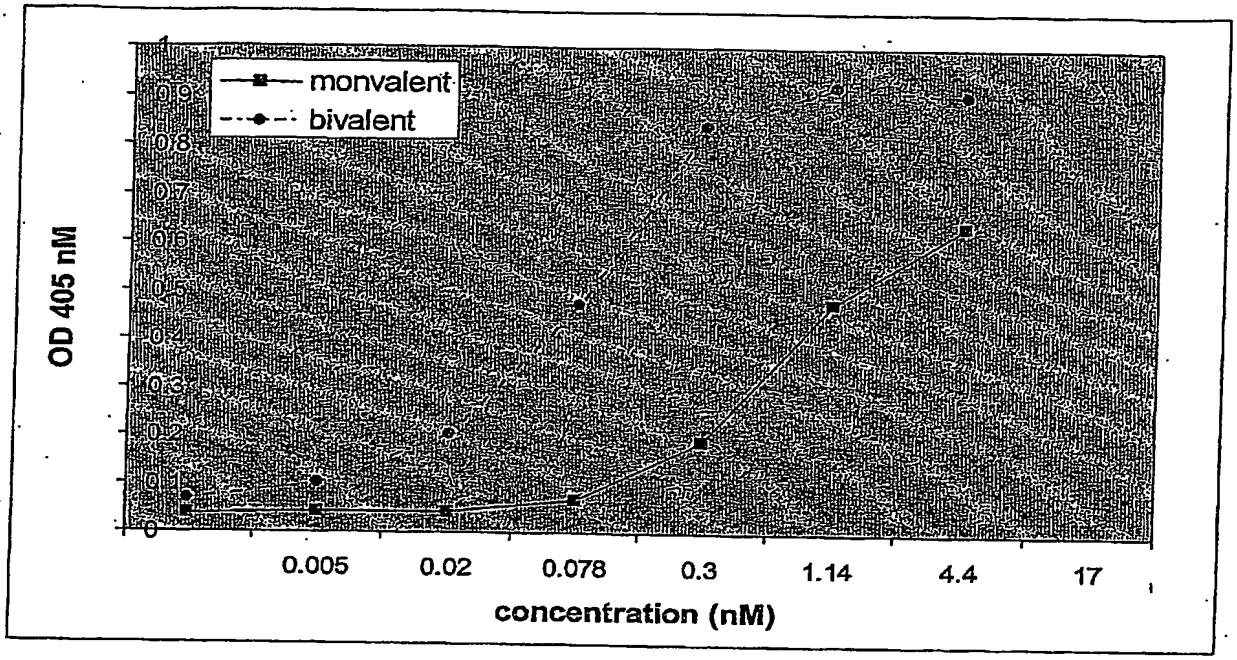

monvalent
bivalent
OD 405 nM
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.005
0.02
0.078
0.3
1.14
4.4
17
concentration (nM)

FIGURE 6

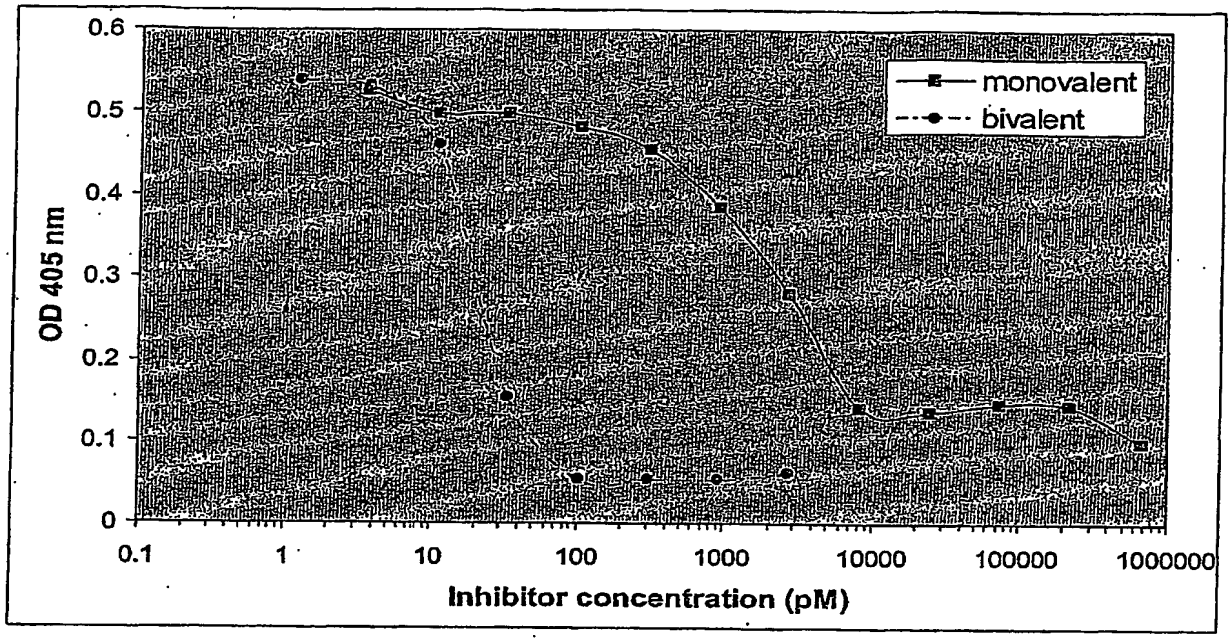

0.6
0.5
0.4
0.3
0.2
0.1
0
OD 405 nm
0.1
1
10
100
1000
10000
100000
1000000
Inhibitor concentration (pM)
monovalent
bivalent

FIGURE 7

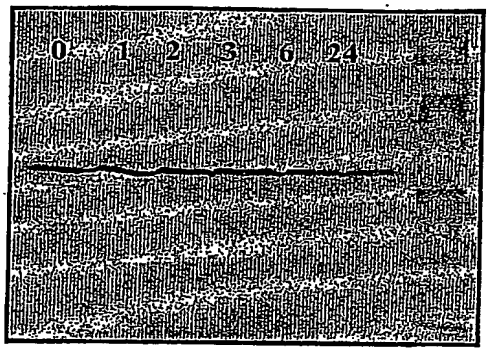


FIGURE 8

```
                        FR1                          CDR1            FR2
VHH#3          QVQLQDSGGGLVQAGGSLRLSCAVSGR TFSAHS--VYTMG WFRQAPGKEREFVA
VHH#2          QVQLQESGGGLVQPGGSLRLSCAASGR TFSDHSGYTYTIG WFRQAPGKEREFVA
VHH#1          QVQLQESGGGLVQPGGSLRLSCATSGF DFS--VSWMY--- WVRQAPGKGLEWVS
VHH#13         QVQLQESGGGLVQPGGSLRLSCATSGF TFS--DYWMY--- WVRQAPGKGLEWVS
VHH#9          QVQLQESGGGLVQPGGSLRLSCAASGS IFRVNA-----MG WYRQVPGNQREFVA
               ***** ******* ********* **   *            * ** **   * *

                    CDR2                          FR2
VHH#3          RIYWSSANTYYADSVKG RFTISRDNAKNTVDLLMNSLKPEDTAVYYCAA
VHH#2          RIYWSSGNTYYADSVKG RFAISRDIAKNTVDLTMNNLEPEDTAVYYCAA
VHH#1          EINTNGLITKYVDSVKG RFTISRDNAKNTLYLQMDSLIPEDTALYYCAR
VHH#13         TVNTNGLITRYADSVKG RFTISRDNAKYTLYLQMNSLKSEDTAVYYCTK
VHH#9          -IITSGDNLNYADAVKG RFTISTDNVKKTVYLQMNVLKPEDTAVYYCNA
                        * * *** ** ** *  * *  * *  *  **** ***

                    CDR3          FR4        Hinge
VHH#3          RDGIPTSRTVGSYNY WGQGTQVTVSS EPKTPKPQP
VHH#2          RDGIPTSRSVESYNY WGQGTQVTVSS EPKTPKPQP
VHH#1          ---------SPSGSF RGQGTQVTVSS EPKTPKPQP
VHH#13         -VVPPYSDDSRTNAD WGQGTQVTVSS EPKTPKPQP
VHH#9          --ILQTSRWSIPSNY WGQGTQVTVSS EPKTPKPQP
               ********** *********
```

FIGURE 9

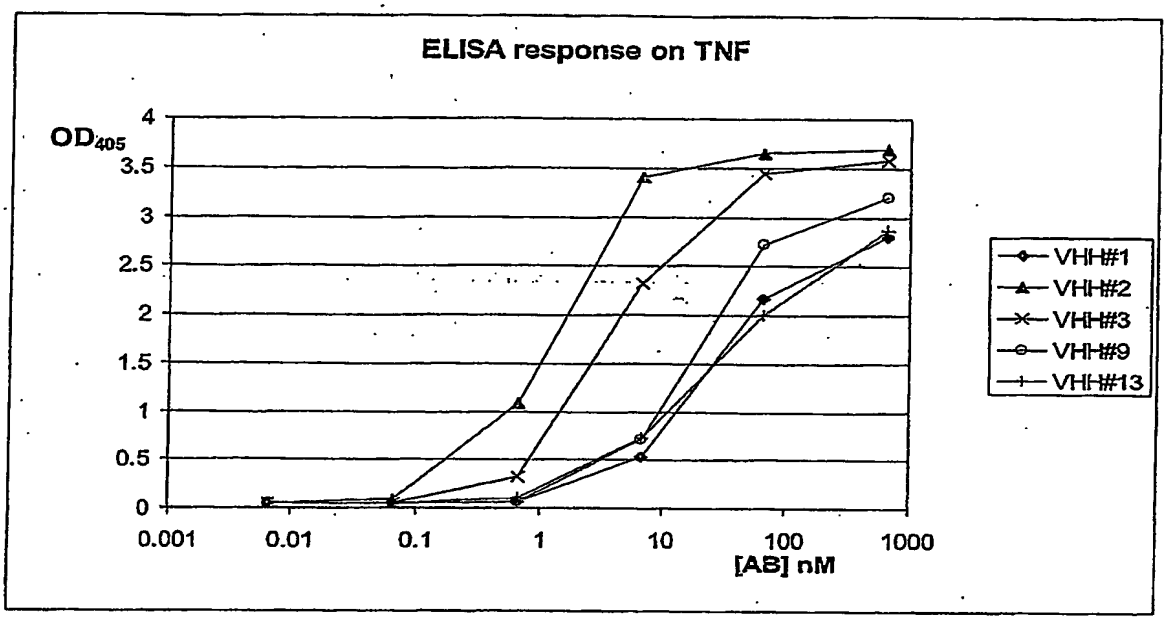

ELISA response on TNF
OD405
4
3.5
3
2.5
2
1.5
1
0.5
0
0.001
0.01
0.1
1
10
100
1000
[AB] nM
VHH#1
VHH#2
VHH#3
VHH#9
VHH#13

FIGURE 10

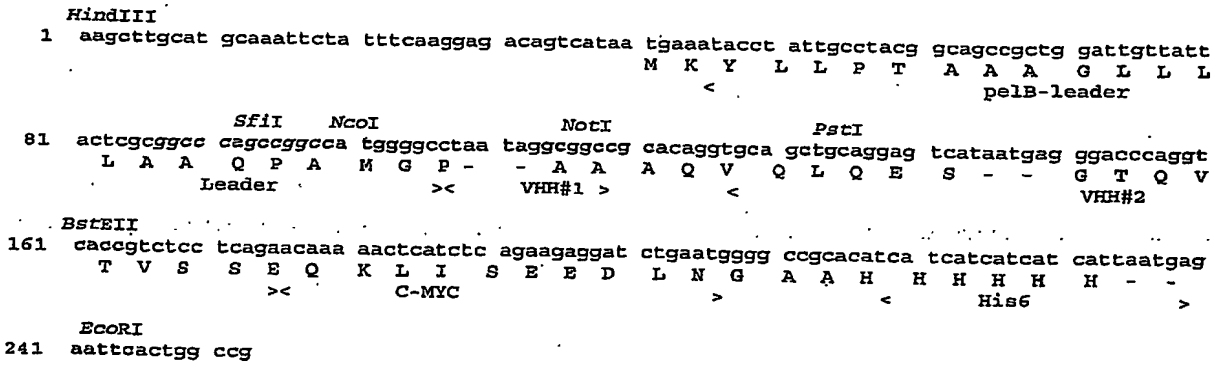

HindIII
1 aagcttgcat gcaaattcta tttcaaggag acagtcataa tgaaatacct attgcctacg gcagccgctg gattgttatt
M K Y L L P T A A A G L L L
pelB-leader
SfiI NcoI NotI PstI
81 actcgcggcc cagccggcca tggggcctaa taggcggccg cacaggtgca gctgcaggag tcataatgag ggacccaggt
L A A Q P A M G P - - A A A Q V Q L Q E S - - G T Q V
Leader >< VHH#1 > < VHH#2
BstEII
161 caccgtctcc tcagaacaaa aactcatctc agaagaggat ctgaatgggg ccgcacatca tcatcatcat cattaatgag
T V S S E Q K L I S E E D L N G A A H H H H H H - -
>< C-MYC > < His6 >
EcoRI
241 aattcactgg ccg

FIGURE 11

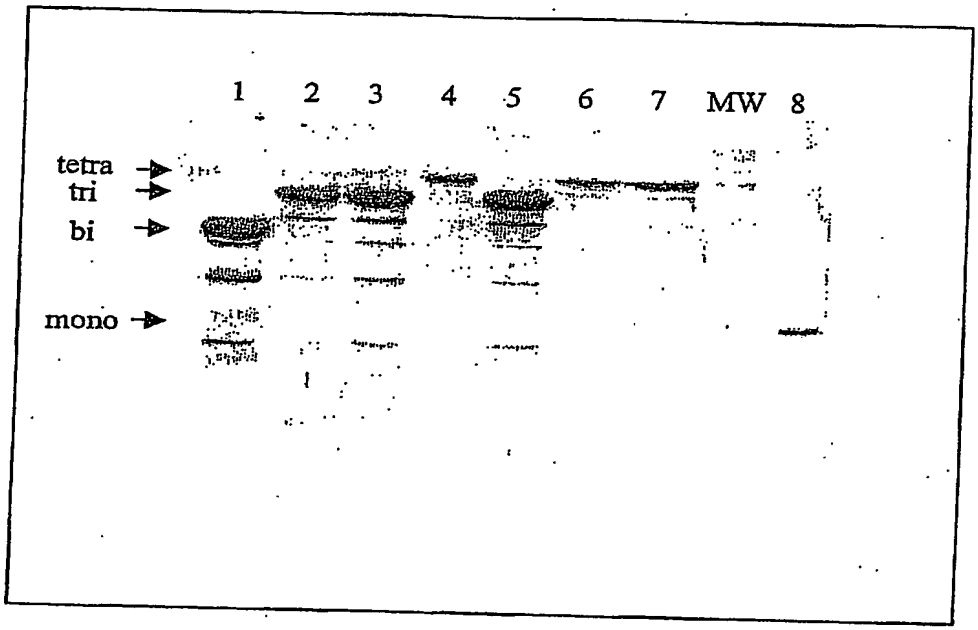


FIGURE 12

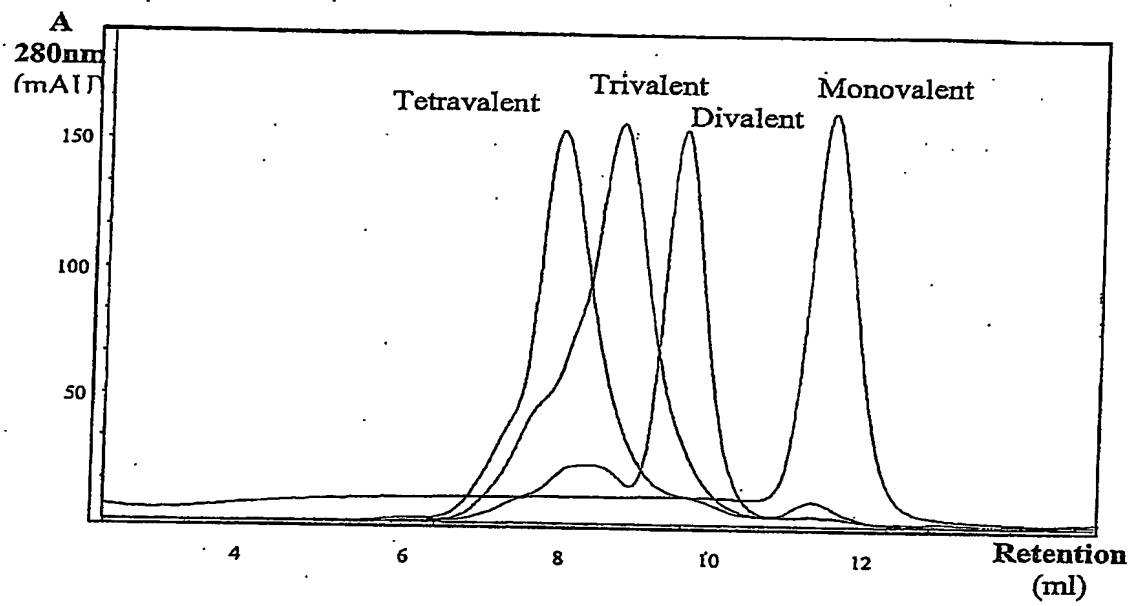

A
280nm
(mAU)
150
100
50
Tetravalent
Trivalent
Divalent
Monovalent
4
6
8
10
12
Retention
(ml)

FIGURE 13

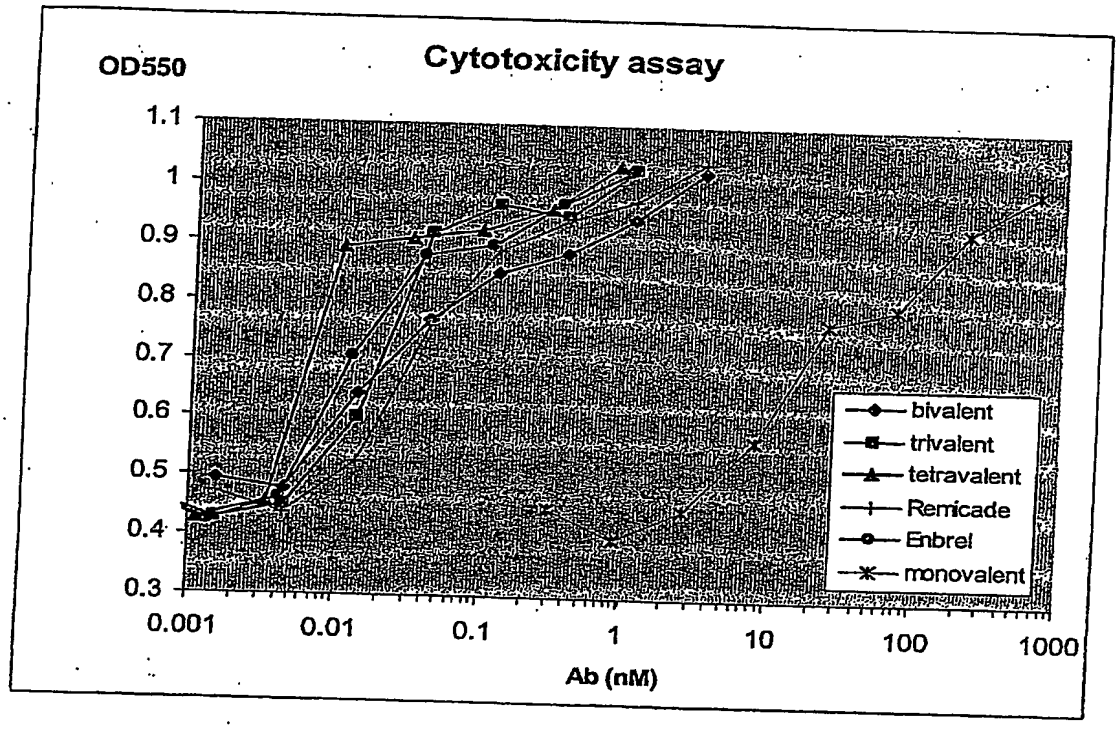

Cytotoxicity assay
OD550
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.001
0.01
0.1
1
10
100
1000
Ab (nM)
bivalent
trivalent
tetravalent
Remicade
Enbrel
monovalent

FIGURE 14

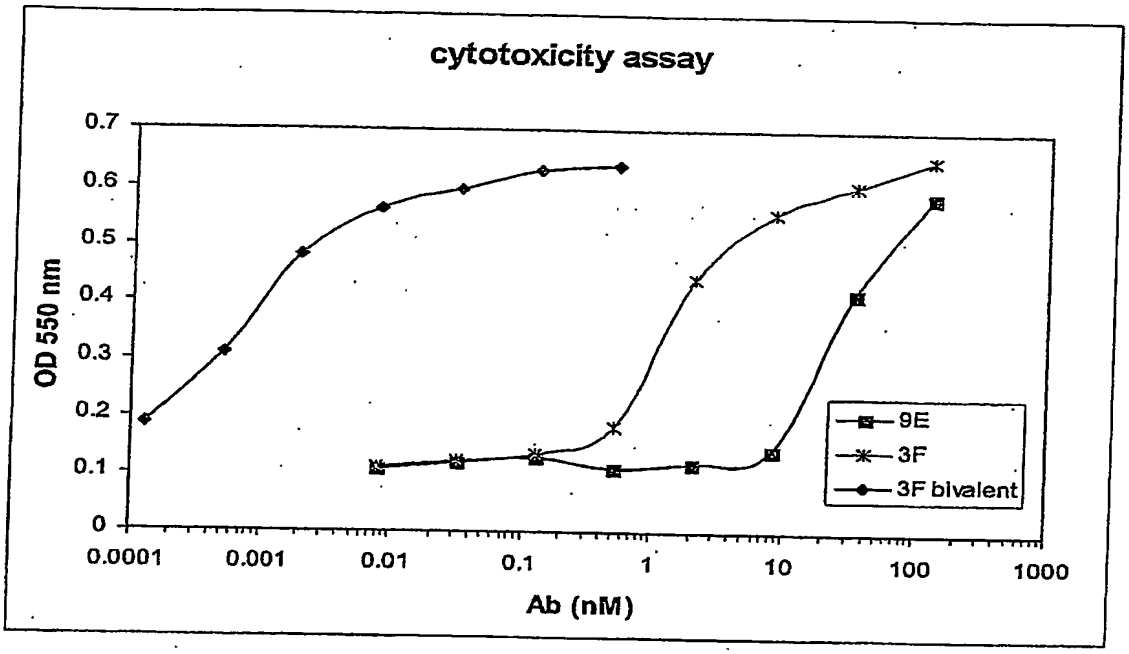
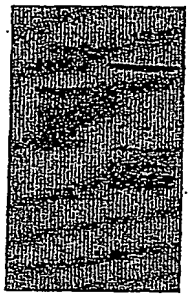
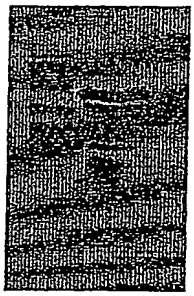

cytotoxicity assay
OD 550 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.0001
0.001
0.01
0.1
1
10
100
1000
Ab (nM)
9E
3F
3F bivalent

FIGURE 15

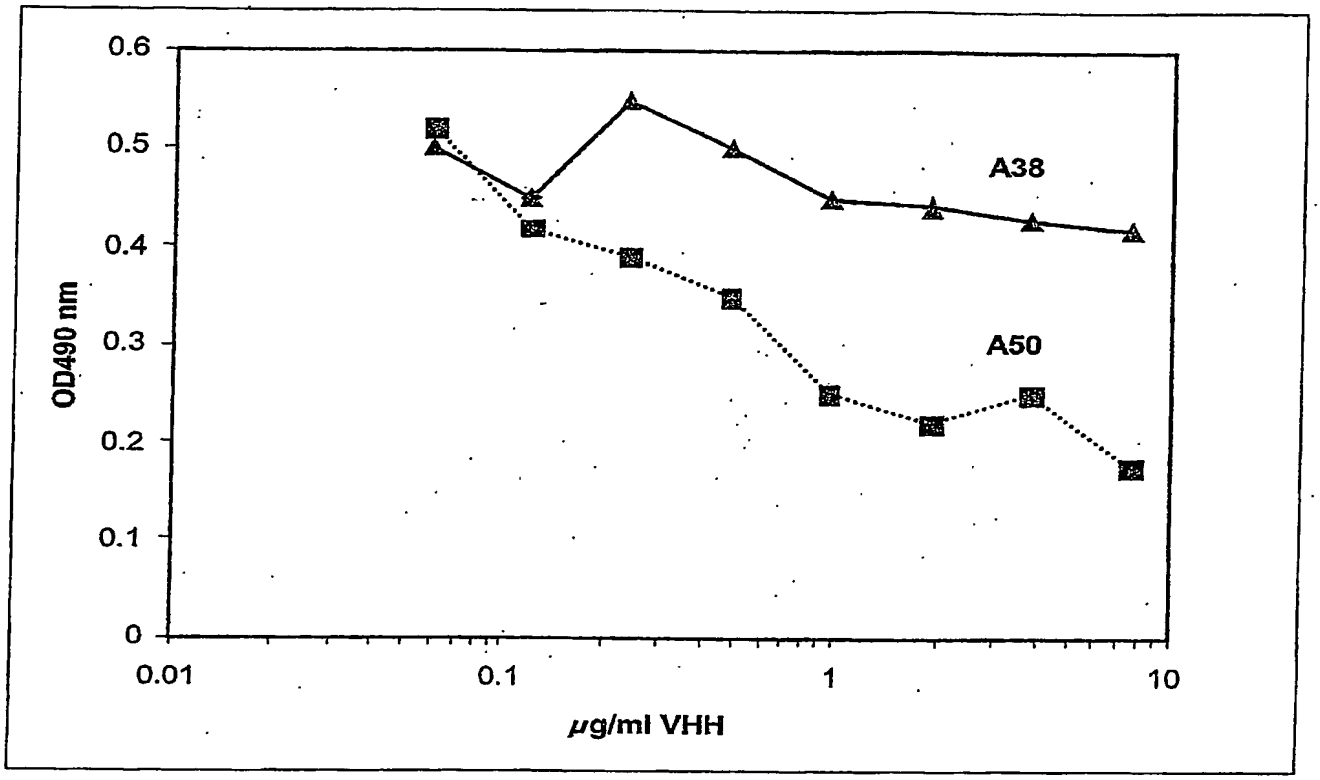


A1 domain



A3 domain

FIGURE 16



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